ADVANCES AND CHALLENGES IN NANOMEDICINE

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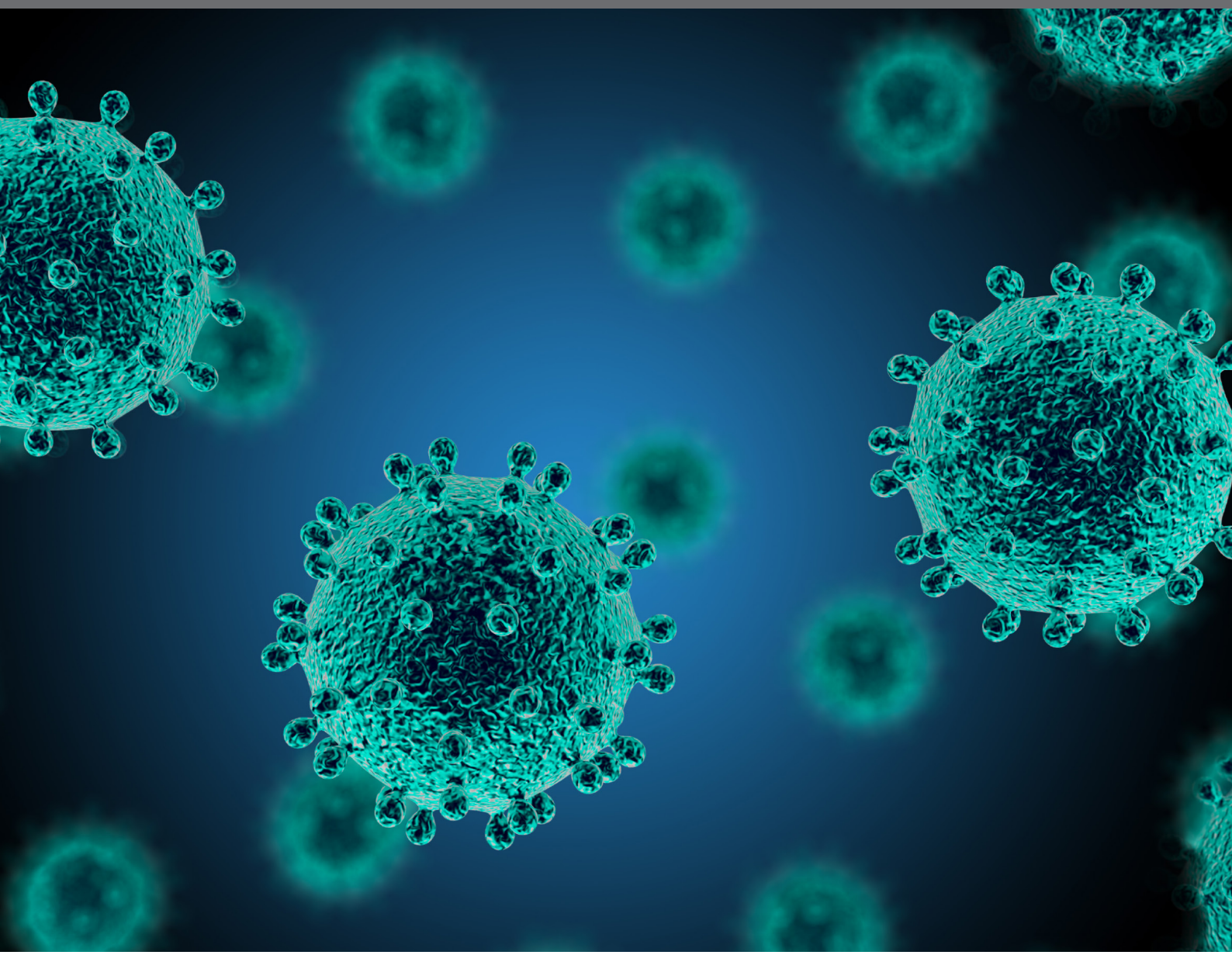

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ADVANCES AND CHALLENGES IN NANOMEDICINE

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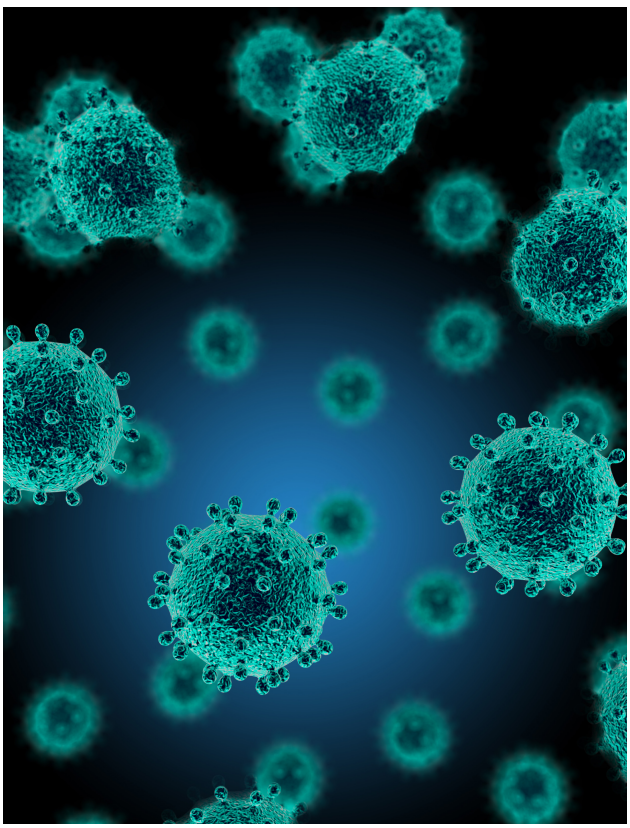

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Nanotechnology is a multidisciplinary field that is revolutionizing the way we detect and treat damage to the human body. Nanomedicine applies nanotechnology to highly specific medical interventions for the prevention, diagnosis, and treatment of diseases. They are increasingly being used to overcome biological barriers in the body to improve the way we deliver compounds to specific tissues and organs. In particular, nanomedicines have been shown to be beneficial for stabilizing therapeutic compounds, overcoming obstacles to cellular and tissue uptake, and improving biodistribution of compounds to target sites in vivo. Nanomedicines have demonstrated significant therapeutic advantages for a multitude of biomedical applications, however the clinical translation of these nanotechnology platforms has not progressed as quickly as the plethora of positive results would have suggested.

Understanding the advances in nanomedicine to date and the challenges that still need to be overcome, will allow future research to improve on existing platforms and to address the current translational and regulatory limitations.

This eBook "Advances and Challenges in Nanomedicine" has brought together experts in the fields of nanomedicine, nanotechnology, nanotoxicology, pharmaceutics, manufacturing, and translation to discuss the application of nanotechnology to drug delivery. This information is presented as original research, opinion, perspective, and review articles. The goal of this eBook is to generate collaborative discussion on the current status, general trends, challenges, strategies, and future direction of pharmaceutical nanotechnology, as well as highlight current and emerging nanoparticulate platforms with potential medical applications.

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EDITORIAL
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Editorial: Advances and Challenges in Nanomedicine

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Keywords: nanomedicine, nanotechnology, nanoparticles, targeted drug delivery, translation

Editorial on the Research Topic

Advances and Challenges in Nanomedicine

The use of nanotechnology in medicine has the potential to have a significant impact on human health by improving the diagnosis, prevention and treatment of diseases. Nanomedicines typically encapsulate therapeutic and/or imaging compounds in submicrometer-sized carrier materials. In the last several decades, the application of nanomedicine for clinical purposes has received significant attention from academia, researchers, government, funding agencies, and regulatory bodies (Allen and Cullis, 2004; Sercombe et al., 2015; Hare et al., 2017; Hua et al.). Nanomedicines are generally intended to increase the therapeutic index of compounds by allowing more efficient delivery to the target site to enhance therapeutic efficacy and/or by minimizing accumulation in healthy body sites to reduce toxicity. Nanoencapsulation can also protect therapeutics from degradation in biological environments and can provide solubilization (Talekar et al., 2015; Mishra et al., 2017; Shajari et al., 2017). This e-Book focuses on articles that discuss the advances and challenges in the nanomedicine field across a broad range of topics. A brief summary of each article is provided below.

Nanomedicines can have a combination of chemical, physical, and biological properties that influences their *in vivo* behavior.

- Arms et al. addressed the principles and methodology of the available techniques for evaluating *in vivo* biodistribution of nanoparticles (Arms et al.). They further compared the advantages, limitations and capabilities of the techniques for assessing cellular, whole-organ and real-time accumulation (Arms et al.). Determining the *in vivo* biodistribution of nanoparticles following administration in animals and humans is an important component in the translational assessment of nanomedicines (Arms et al.).
- In addition, Robson et al. discussed the advantages and limitations of available imaging techniques used to evaluate the morphology of liposomal formulations (Robson et al.). Validating and controlling the morphology of nanoparticles is important for clinical translation, however it is generally difficult to control and not well-characterized (Robson et al.).

There are a number of factors that can impose significant obstacles to the clinical translation of nanomedicines, irrespective of whether they are therapeutically beneficial or not.

- Accomasso et al. summarized the current state regarding the safety evaluation of nano-based therapeutics and discussed the importance of risk assessment and risk minimization in the development of nanomedicines (Accomasso et al.). With the rapid growth in the use of nanomaterials for medical applications, the most urgent need is developing and validating novel and practical approaches that are able to determine potential short-term and long-term health risks, including the extrapolation of acute *in vitro* results for the prediction of chronic *in vivo* effects (Accomasso et al.).

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- More broadly, Hua et al. discussed the current trends and challenges in the clinical translation of nanoparticulate nanomedicines, as well as the potential pathways for translational development and commercialization (Hua et al.). Key issues related to the clinical development of nanoparticulate nanomedicines include biological challenges, biocompatibility and safety, large scale manufacturing, government regulations, intellectual property (IP), and overall cost-effectiveness in comparison to current therapies (Hua et al.).

Undoubtedly, the vast majority of nanomedicines in preclinical and clinical development as well as in clinical use are for targeting a wide variety of malignant tumors.

- Yang et al. reviewed the advances and challenges in utilizing exosomes for the delivery of cancer therapeutics (Yang and Wu). Significant progress has been made to overcome major barriers for using exosomes as a delivery system, thereby opening a new promising avenue for cancer treatment (Yang and Wu).
- Chang et al. highlighted the current understanding and challenges of biologically targeted magnetic hyperthermia to induce cancer cell death and potentially improve the effectiveness and safety of hyperthermia (Chang et al.). Application of an alternating magnetic field following administration of cancer-targeting magnetic nanoparticles that accumulate in the tumor allows preferential heating of malignant cancer cells (Chang et al.). Despite promising results in preclinical studies, there are a number of challenges that still need to be addressed before this technique can progress to the clinic (Chang et al.).
- Furthermore, Buss et al. discussed how nanotechnology can help overcome current obstacles for the treatment of bladder cancer. This includes how it can be used in non-muscle-invasive urothelial bladder cancer to facilitate combination chemotherapeutic and BCG (mycobacterium bovis bacillus Calmette–Guerin) immunotherapies (Buss et al.).

Modulation of the tumor microenvironment has recently emerged as an important strategy to improve the delivery of nanomedicines to tumors, given the importance of cancer-associated cells in tumor growth and metastasis.

- Zhang et al. discussed the existing approaches and strategies for modulating the tumor microenvironment to improve tumor perfusion (Zhang et al.). This enables accumulation of nanomedicines at the tumor site, facilitates extravasation of nanomedicines for improving transvascular transport, and enhances interstitial transport for optimizing the biodistribution of nanomedicines (Zhang et al.). These strategies may provide an opportunity for the development of novel combination chemotherapeutic regimens and reassessment of previously suboptimal compounds (Zhang et al.).
- Correspondingly, Harrison et al. addressed the advances and challenges of nucleic acid delivery of nanoparticles to the tumor microenvironment (Harrison et al.). Despite the development of various nanoparticle platforms to overcome nucleic acid delivery hurdles, several challenges still exist for effective tumor delivery (Harrison et al.). One such challenge has been the accumulation of nanoparticles in non-cancer cells within the tumor microenvironment, which has recently opened up novel therapeutic applications for nanoparticles (Harrison et al.).

The application of nanomedicine-based therapies for drug targeting to non-cancer conditions has also increased in recent years.

- Remiao et al. presented recent developments in nanotechnology to overcome impairments still faced by medically assisted reproductive technology (e.g. multiple pregnancy, ovary stimulation, and genetic disorders) and new perspectives for the further use of nanotechnology in reproductive medicine (Remião et al.). The application of nanotechnology approaches to reproductive medicine have provided strategies to improve diagnosis and increase specificity and sensitivity (Remião et al.).
- Fang et al. showed that liposome-encapsulated baicalein may have the potential to improve wound healing and restore skin structure after skin injury (Fang et al.). The study demonstrated that liposome-encapsulated baicalein can inhibit adipogenic differentiation medium (ADM)-induced lipid accumulation and extracellular matrix formation in Hs68 fibroblasts through the suppression of lipogenesis enzymes and inflammatory responses (Fang et al.).
- In addition, Yan et al. have elucidated the possible pathways for layered double hydroxide (LDH) nanoparticles to enhance antigen (cross)-presentation on immune cells as adjuvants for protein vaccines (Yan et al.). Nanoparticles have been intensively investigated as adjuvants in new generation vaccines, however how these nanoparticles provoke immune responses is not well-understood (Yan et al.). This research would help to understand the nanoparticle adjuvant mechanism and further assist the design of new specific nanoparticles as more efficient nano-adjuvants (Yan et al.).

SUMMARY

Overall, nanomedicine has the potential to revolutionize the way we detect and treat damage to the human body. Although nanomedicines have demonstrated significant therapeutic advantages for a multitude of medical applications, their translation has not progressed as rapidly as the plethora of positive preclinical results would have suggested (Luxenhofer et al., 2014; Sercombe et al., 2015; Hare et al., 2017; Hua et al.). The experimental development of nanomedicines is continually progressing at a fast pace, however significant challenges still exist in promoting these platforms into clinically feasible therapies. Therefore, continued translational success will require communication and collaboration between experts involved in all stages of pharmaceutical development of nanotechnologies,

including pharmaceutical design and manufacturing, cellular interactions and toxicology, as well as preclinical and clinical evaluation (Hua et al.).

AUTHOR CONTRIBUTIONS

SH drafted the manuscript. SH and SW critically reviewed the manuscript for important intellectual content.

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REFERENCES

Allen, T. M., and Cullis, P. R. (2004). Drug delivery systems: entering the mainstream. *Science* 303, 1818–1822. doi: 10.1126/science.1095833

Hare, J. I., Lammers, T., Ashford, M. B., Puri, S., Storm, G., and Barry, S. T. (2017). Challenges and strategies in anti-cancer nanomedicine development: an industry perspective. *Adv. Drug Deliv. Rev.* 108, 25–38. doi: 10.1016/j.addr.2016.04.025

Luxenhofer, R., Barz, M., and Schillmeier, M. (2014). Quo vadis nanomedicine? *Nanomedicine* 9, 2083–2086. doi: 10.2217/nnm.14.140

Mishra, D. K., Balekar, N., and Mishra, P. K. (2017). Nanoengineered strategies for siRNA delivery: from target assessment to cancer therapeutic efficacy. *Drug Deliv. Transl. Res.* 7, 346–358. doi: 10.1007/s13346-016-0352-5

Sercombe, L., Veerati, T., Moheimani, F., Wu, S. Y., Sood, A. K., and Hua, S. (2015). Advances and challenges of liposome assisted drug delivery. *Front. Pharmacol.* 6:286. doi: 10.3389/fphar.2015.00286

Shajari, N., Mansoori, B., Davudian, S., Mohammadi, A., and Baradaran, B. (2017). Overcoming the challenges of siRNA delivery: nanoparticle strategies. *Curr. Drug Deliv.* 14, 36–46. doi: 10.2174/1567201813666160816105408

Talekar, M., Tran, T. H., and Amiji, M. (2015). Translational nano-medicines: targeted therapeutic delivery for cancer and inflammatory diseases. *AAPS J.* 17, 813–827. doi: 10.1208/s12248-015-9772-2

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REVIEW
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Advantages and Limitations of Current Techniques for Analyzing the Biodistribution of Nanoparticles

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Nanomedicines are typically submicrometer-sized carrier materials (nanoparticles) encapsulating therapeutic and/or imaging compounds that are used for the prevention, diagnosis and treatment of diseases. They are increasingly being used to overcome biological barriers in the body to improve the way we deliver compounds to specific tissues and organs. Nanomedicine technology aims to improve the balance between the efficacy and the toxicity of therapeutic compounds. Nanoparticles, one of the key technologies of nanomedicine, can exhibit a combination of physical, chemical and biological characteristics that determine their *in vivo* behavior. A key component in the translational assessment of nanomedicines is determining the biodistribution of the nanoparticles following *in vivo* administration in animals and humans. There are a range of techniques available for evaluating nanoparticle biodistribution, including histology, electron microscopy, liquid scintillation counting (LSC), indirectly measuring drug concentrations, *in vivo* optical imaging, computed tomography (CT), magnetic resonance imaging (MRI), and nuclear medicine imaging. Each technique has its own advantages and limitations, as well as capabilities for assessing real-time, whole-organ and cellular accumulation. This review will address the principles and methodology of each technique and their advantages and limitations for evaluating *in vivo* biodistribution of nanoparticles.

Keywords: nanoparticles, nanomedicine, biodistribution, *in vivo*, imaging, techniques, advantages, limitations

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INTRODUCTION

Nanomedicine is the application of nanotechnology for the diagnosis, prevention and treatment of diseases. Nanomedicines are submicrometer-sized carrier materials (nanoparticles) designed to improve the biodistribution of encapsulated compounds by delivering them more effectively and more selectively to the pathological site (site-specific drug delivery) and/or by guiding them away from potentially endangered healthy tissues (site-avoidance drug delivery) (Lammers et al., 2012). This technology aims to improve the balance between the efficacy and the toxicity of therapeutic compounds (Lammers et al., 2012). Nanoparticles can exhibit a combination of physical (e.g., size, shape, lamellarity and homogeneity),

chemical (e.g., composition, surface charge, surface coating and phase transition temperature), and biological (e.g., encapsulated compounds and conjugated surface ligands) characteristics that determine their *in vivo* behavior (Bharali and Mousa, 2010; Robson et al., 2018).

Despite the significant advances in drug delivery technologies and platforms in the last several decades, the clinical translation of nanomedicines has progressed incrementally (Sercombe et al., 2015; Hare et al., 2017). It has been suggested that effective nanomedicine development requires a disease-driven approach, rather than the traditional formulation-driven approach where drug delivery system engineering has been the priority (Hare et al., 2017). This requires a strong understanding of the relationships between biology and technology, including the influence of disease pathophysiology on nanomedicine accumulation, distribution, retention and efficacy, and the correlation between delivery system properties and *in vivo* behavior in animals vs. humans (Hare et al., 2017).

A key component in the translational assessment of nanomedicines is determining the biodistribution of the nanoparticles following *in vivo* administration in animals and humans (Kunjachan et al., 2015). There is a range of techniques available for evaluating nanoparticle biodistribution, including histology, electron microscopy, liquid scintillation counting (LSC), indirectly measuring drug concentrations, *in vivo* optical imaging, computed tomography (CT), magnetic resonance imaging (MRI), and nuclear medicine imaging. Each technique has its own advantages and limitations, as well as capabilities for assessing real-time, whole-organ and cellular accumulation (**Figure 1**). This review will address the principles and methodology of each technique and their advantages and limitations for evaluating *in vivo* biodistribution of nanoparticles.

HISTOLOGY

Microscopic visualization of nanoparticles in tissue sections is one of the common techniques used to assess biodistribution following *in vivo* administration in animals. This technique relies on histological processing of tissues to examine the association of nanoparticles with the cellular microenvironment under a microscope—typically light and fluorescence microscopy. In order to assess nanoparticle biodistribution, the organs or tissues of interest are harvested at set time points following *in vivo* administration in animals and undergo either conventional paraffin processing or cryostat processing of frozen or fixed tissues. The choice between the methods depends on the composition of the nanomedicine, as paraffin processing involves the samples being dehydrated, cleared (also called delipidation) and infiltrated. More specifically, water is removed from the specimen in successive stages using increasing concentrations of alcohol. A clearing agent, such as xylene, is used in the last phase to remove the alcohol and tissue lipids in the specimen, thereby allowing infiltration of an embedding agent (e.g., paraffin wax or cryogenic media) (Alturkistani et al., 2016). As this process involves the use of lipid soluble solvents, nanoparticles composed of materials that are easily degraded by these solvents (e.g., liposomes, solid lipid nanoparticles, and micelles) should only undergo cryostat processing of frozen sections.

For paraffin processing, tissues of interest are immediately fixed with chemicals (commonly formalin) to preserve structural integrity and prevent cell degradation (autolysis and putrefaction), prior to embedding and sectioning using a microtome. This process, however, can damage proteins in the tissue and can also denature them to a certain extent, which may include protein or peptide-based ligands conjugated to the surface of nanoparticles (Titford, 2009). On the other hand, frozen tissues may be fixed or unfixed and are sectioned using a microtome mounted in a refrigeration device known as a cryostat. Sections are then mounted on a glass slide and can be stained to enhance visualization of the nanoparticles and/or microscopic anatomy of cells and tissues. A variety of histological stains can be used to differentiate between biological structures, with hematoxylin and eosin (H&E) being the most common (Titford, 2009; Alturkistani et al., 2016). H&E staining provides excellent visualization of nuclei (stained purple) and cytoplasmic details (stained pink) within cells (Titford, 2009; Alturkistani et al., 2016). It is important to choose a stain to label biological structures that will not affect the nanoparticles themselves. Immunohistochemistry or co-labeling can also be used to visualize other aspects of the tissues, such as blood vessels, to allow appropriate orientation and evaluation of the cellular biodistribution of nanoparticles in tissue sections.

Conventional histopathology staining methods can be used to detect the biodistribution of certain types of nanoparticles. In particular, clusters of nanoparticles >200 nm in size can be visualized by light microscopy in tissue sections based on resolution limitations (Ostrowski et al., 2015; Robson et al., 2018). For example, the biodistribution of ultrasmall and small superparamagnetic iron oxide (USPIO and SPIO, respectively) nanoparticles that were injected intraperitoneally into C57BL/6 mice were studied histologically by measuring iron-positive areas (μm^2) in representative paraffin-embedded tissue sections of organs stained with Prussian blue (Tsuchiya et al., 2011; Pham et al., 2018). Similarly, cationic stains such as Alcian blue have been used to stain the negatively charged sulfate groups embedded within organic dendritic polyglycerol sulfate (dPGS) nanoparticles. Holzhausen et al were able to demonstrate specific localization of dPGS nanoparticles in hepatic Kupffer cells following intravenous injection in mice using Alcian blue in standard histopathological tissue sections (Holzhausen et al., 2013). Single-walled carbon nanotubes (SWCNT) labeled with colloidal gold have also been visualized as dark deposits on cryostat tissue sections using silver enhancement (Mercer et al., 2008). In addition, the biodistribution of a range of nanoparticles labeled with fluorescent dyes have been visualized in tissues sections using fluorescence microscopy, including silica nanoparticles (Cho et al., 2009) and polymeric micelles (Asem et al., 2016).

In terms of advantages, histology is a relatively cost-effective technique for assessing nanoparticle biodistribution and allows for the study of large tissue sections (**Table 1**). In comparison to other available techniques, histology can be used to study the specific accumulation and association of nanoparticles within a

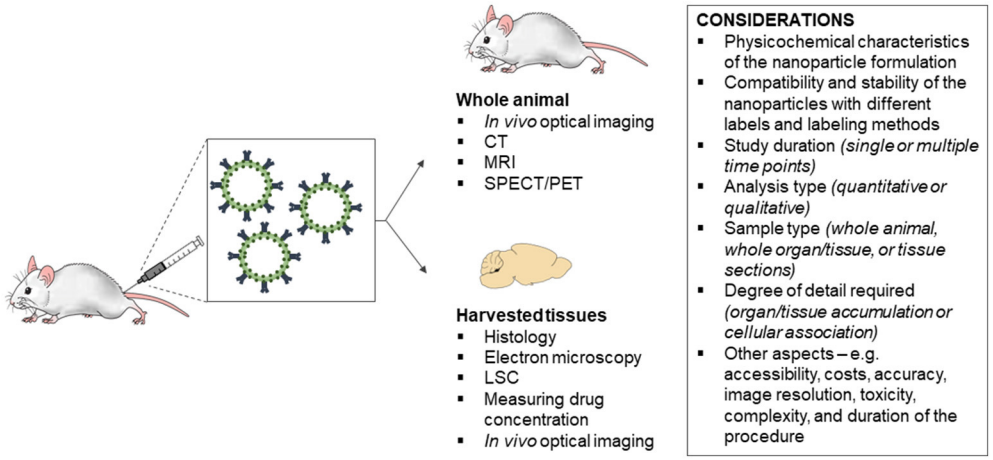

FIGURE 1 | Considerations for the choice of technique for evaluating the *in vivo* biodistribution of nanoparticles. CT, computed tomography; MRI, magnetic resonance imaging; SPECT, single photon emission computed tomography; PET, positron emission tomography; LSC, liquid scintillation counting.

cellular context. This technique also does not require exposure to ionizing radiation or contrast agents. However, histology is generally considered a qualitative method when assessing nanoparticle biodistribution and a number of limitations should be considered when approaching this technique. Light and fluorescence microscopy have generally low resolution compared to other microscopy techniques and are unable to image individual nanoparticles in the lower nanometer range, especially in tissues (Robson et al., 2018). In addition, a limited number of tissue sections (5–50 μm thickness) are typically chosen to evaluate and approximate biodistribution in each organ, simply due to the sheer number of tissue sections that can be attained from each organ. This may affect the results as not all sections are examined; therefore, appropriate sampling methods should be utilized to provide a more reliable representation of nanoparticle biodistribution in the whole organ. Histology is also a time-consuming and laborious technique. Although cryostat sectioning may be faster to prepare than paraffin-embedded tissue sections, the freezing process may negatively affect tissue structures and resolution, especially when using light microscopy. Furthermore, histology is susceptible to human error during slide preparation and analysis, and the identification of specific cell types can be difficult. In particular, the detection of organic nanoparticles in tissue sections often poses a particular challenge due to their closer similarities with biomolecules (Holzhausen et al., 2013). With regards to fluorescence imaging, the labeling of nanoparticles with fluorescent dyes may affect their physicochemical properties and subsequent *in vivo* behavior (Robson et al., 2018). Photobleaching of fluorescent dyes is another concern (Robson et al., 2018), especially when the fluorescent-labeled nanoparticles are likely to have some exposure to light during the study from *in vivo* administration to tissue harvesting and processing. This can result in a diminished fluorescent signal. Tissue autofluorescence is also a significant issue that needs to be addressed with appropriate control groups if using this technique. Autofluorescence occurs in most tissues and leads to a reduction in the signal detection sensitivity, which interferes with the accuracy of the results (Koo et al., 2006).

ELECTRON MICROSCOPY

Electron microscopy analysis of tissue samples can provide detailed information of the biodistribution of nanoparticles under very high magnification (Mayhew et al., 2009). This technique uses a beam of electrons focused onto the surface of the sample by various electromagnetic lenses. The electrons are scattered by the sample and are then refocused and magnified by a further series of electromagnetic lenses in the imaging column to produce a projected image (Mayhew et al., 2009; Robson et al., 2018). There are a number of different types of electron microscopes, with transmission electron microscopy (TEM), scanning electron microscopy (SEM) and variations of the two techniques having been utilized for this application. In comparison to TEM, in which the electron beam crosses the sample where it is then focused by the objective lens to form an image, SEM utilizes an electron beam that is scanned across or over a sample (rather than through a sample) and imaging is performed by mapping signal intensity synchronously with the scan to produce a magnified image of an object (Garcia-Negrete et al., 2015; Robson et al., 2018). Typically, tissue samples are fixed with chemicals (commonly formalin) and then undergo dehydration with serial alcohol and propylene oxide, prior to embedment in embedding resin (e.g., glycidether 100, EPON 812, Embed 812).

Electron microscopy has predominantly been used to determine the cellular association of nanoparticles *in vitro* (Schrand et al., 2010; Plascencia-Villa et al., 2012; Brown and Hondow, 2013; Goldstein et al., 2014), with only limited studies using this technique to evaluate nanoparticle biodistribution following *in vivo* administration (Muhlfeld et al., 2007; Mayhew et al., 2009; Jong et al., 2010; Kempen et al., 2013; Garcia-Negrete et al., 2015). For example, Jong et al. (2010) evaluated

TABLE 1 | Summary of current techniques for analyzing the biodistribution of nanoparticles.

Technique	Advantages and limitations for the evaluation of nanoparticle biodistribution
Histology	**ADVANTAGES** • Relatively cost-effective technique • Generally considered a qualitative method of biodistribution • Allows for the study of large tissue sections • Can be used to study the specific cellular association of nanoparticles within tissues • Does not require exposure to ionizing radiation or contrast agents **DISADVANTAGES** • Light and fluorescence microscopy provide low resolution imaging of nanoparticles in tissue sections • Nanoparticle biodistribution in a whole organ is typically approximated by evaluating a limited number of tissue sections • Time-consuming and laborious technique • Freezing process for cryostat sectioning may affect tissue structure and resolution, especially when using light microscopy • Susceptible to human error during slide preparation and analysis • Identification and differentiation between certain cell types and nanoparticles in tissue sections can be difficult • Labeling of nanoparticles with fluorescent dyes for fluorescence imaging of histology sections may affect their physicochemical properties and subsequent *in vivo* behavior • Photobleaching of fluorescent-labeled nanoparticles can occur following exposure to light during *in vivo* administration to tissue harvesting and processing • Tissue autofluorescence is a significant issue that needs to be addressed with appropriate control groups if using fluorescence imaging
Electron microscopy	**ADVANTAGES** • Can provide detailed information of the biodistribution of nanoparticles under very high magnification • Allows visualization of the accumulation of nanoparticles in cells and the localization of nanoparticles in cellular organelle • Generally considered a semi-quantitative method • Predominantly been used to determine the cellular association of nanoparticles *in vitro*, with only limited studies using this technique to evaluate nanoparticle biodistribution following *in vivo* administration **DISADVANTAGES** • More expensive technique than standard histology • Not capable of evaluating large tissue sections • Time-consuming technique • Nanoparticle biodistribution in a whole organ is typically approximated by evaluating a limited number of ultra-thin tissue sections • Relatively high numbers of nanoparticles need to be administered • An additional identification technique may also be necessary for a positive identification of the nanomaterial in tissues and cells • Characterization of soft materials can be affected by the high-voltage electron beams • Burn-in spots can form on the image to create artifacts • Sample preparation method will not be suitable for all nanoparticles
Liquid scintillation counting (LSC)	**ADVANTAGES** • Sensitive, specific and quantitative technique • LSC can determine nanoparticle biodistribution at the tissue or organ level **DISADVANTAGES** • This technique can be laborious, especially with the need to treat and solubilize the harvested tissues prior to LSC analysis • May not be an accurate reflection of whole organ biodistribution if a small portion of an organ is collected for LSC • LSC does not provide any information regarding specific cellular association or accumulation of nanoparticles in tissues • Quality and reproducibility of the data will depend on the choice of the cocktail as well as on the sample composition, volume, temperature, and counting device
Measurement of drug concentration in tissues	**ADVANTAGES** • Quantitative measure of biodistribution that can be used to analyze whole or partial tissue samples. • Can be useful as a secondary quantitative measure to support the biodistribution results attained from qualitative techniques • Does not involve exposure to ionizing radiation, incorporation of imaging molecules to nanoparticles, or the administration of contrast agents to enhance imaging outcomes **DISADVANTAGES** • This is an indirect technique that more specifically measures payload biodistribution and may provide unreliable results if the compound prematurely dissociates from the nanoparticles following *in vivo* administration • Accurate measurement of drug concentration is highly dependent upon the quality of the tissue preparation and extraction procedure, which can be time-consuming and laborious • Unable to provide information on real-time biodistribution across time points in animals
In vivo optical imaging	**ADVANTAGES** • Direct and non-invasive technique that is relatively simple to conduct • Fast image acquisition times • Does not require exposure to ionizing radiation • Imaging can be performed in real-time and over multiple time points

(Continued)

TABLE 1 | Continued

Technique	Advantages and limitations for the evaluation of nanoparticle biodistribution
	• Can determine nanoparticle biodistribution at the tissue or organ level • Images produced tend to have high sensitivity and enhanced spatial and temporal resolution • Generally considered a qualitative measure of biodistribution **DISADVANTAGES** • Can have limited tissue penetration (<1 cm) and is prone to attenuation with increased tissue depth • Relatively low spatial resolution compared to CT and MRI • Labeling of nanoparticles with fluorophores may alter their physicochemical properties and *in vivo* behavior • Many fluorophores can undergo photobleaching during the procedure, which affects their sensitivity to imaging • Tissue autofluorescence is a significant issue that can affect the interpretation of results, therefore fluorophores should have higher signal-to-background ratios • Does not provide any information regarding specific cellular association or accumulation of nanoparticles in tissues • It cannot visualize individual nanoparticles, but instead measures broader fluorescence intensity
Computed tomography (CT)	**ADVANTAGES** • Produces reliable and high-resolution images for assessing the biodistribution of nanoparticles • It has no tissue penetration limits and relatively quick image acquisition times • Generally considered a qualitative measure of biodistribution • Can determine nanoparticle biodistribution at the tissue or organ level • Biodistribution of nanoparticles can be assessed in real-time and over multiple time points **DISADVANTAGES** • Requires exposure to ionizing radiation • Does not provide any information regarding specific cellular association of nanoparticles • Often requires the administration of contrast imaging agents to enhance visualization and differentiation among different types of tissues • Potential interference when nanoparticles labeled with contrast agents are used in conjunction with other contrast imaging agents to improve anatomical and tissue imaging • The detection limit of nanoparticle contrast agents is less sensitive compared to other modalities, such as nuclear imaging • Incorporation of contrast agents in nanoparticles may alter their physicochemical properties and *in vivo* behavior
Magnetic resonance imaging (MRI)	**ADVANTAGES** • Direct and non-invasive technique • Does not involve exposure to ionizing radiation • Produces high spatial resolution images compared to other techniques such as optical or radionuclide imaging • Provides better soft tissue contrast than CT and can differentiate better between fat, water, muscle, and soft tissue • Not limited by tissue depth (unlimited penetration) • Can determine nanoparticle biodistribution at the tissue or organ level • Biodistribution of nanoparticles can be assessed in real-time and over multiple time points **DISADVANTAGES** • Relatively more costly technique • Has slow image acquisition and long post-processing times • Much higher amount of contrast agents are generally required, as this technique can suffer from poor sensitivity • Cannot be used in subjects with metallic implants/devices • Incorporation of contrast agents in nanoparticles may alter their physicochemical properties and *in vivo* behavior
Nuclear medicine imaging (PET and SPECT)	**ADVANTAGES** • Quantitative measure of biodistribution • Biodistribution of nanoparticles can be assessed in real-time • Able to image biochemical processes • Not restricted by tissue penetration limits • Highly sensitive technique requiring very small amounts of radiolabels, which minimizes the disruption of cell function and surrounding tissue • PET is much more sensitive than SPECT and provides more radiation event localization data • PET is able to substitute positron-emitters for naturally occurring atoms, thereby enhancing its ability to image molecular events • SPECT can image multiple radionuclide probes simultaneously and is more widely available • SPECT scans are significantly less expensive than PET scans, partly because their radionuclides are simple to prepare, more easily obtained, and typically possess longer half-lives than PET radionuclides **DISADVANTAGES** • Relatively more costly technique • Requires exposure to ionizing radiation • Has slow image acquisition times • Unable to be used for longitudinal studies due to radiolabel decay • Has low spatial resolution and provides a lack of anatomic information, therefore it is often combined with other imaging modalities such as MRI or CT • Type of radionuclide and radiolabeling strategy requires careful consideration, as some nanoparticles may exhibit differing compatibility and imaging effectiveness across the various methods • SPECT has low photon detection efficiency and relatively low resolution compared to PET • PET typically requires a cyclotron or generator

the biodistribution of gold nanoparticles (10 and 250 nm) 24 h post-intravenous injection in rats using TEM. Ultra-thin sections of 50–70 nm were prepared and stained by uranyl acetate and lead citrate. Results showed that 10 nm gold nanoparticles were present in the phagocytic cells of the reticuloendothelial system (RES), whereas 250 nm gold nanoparticles were unable to be detected in any of the organs investigated. This was likely due to the very low number of 250 nm particles that would be theoretically present in one TEM tissue section. In addition, several globular structures of approximately the expected size were found in liver cells and the endothelium of blood vessels in the brain; however, elemental analysis with energy dispersive X-ray (EDX) showed that these structures did not contain gold. This indicates that *in vivo* identification of nanoparticles cannot only depend on the detection of nanosized structures in cells.

More recent studies have used scanning transmission electron microscopy (STEM), which combines the principles of TEM and SEM (Kempen et al., 2013; Garcia-Negrete et al., 2015). STEM requires very thin samples (similar to TEM) and involves scanning a very finely focused beam of electrons across the sample in a raster pattern. This technique allows the use of other signals that cannot be spatially correlated in TEM (e.g., secondary electrons, scattered beam electrons, characteristic X-rays, and electron energy loss) and has improved spatial resolution compared to SEM (Kempen et al., 2013; Garcia-Negrete et al., 2015). For example, Kempen et al. (2013) used STEM to analyze the accumulation and distribution of polyethylene glycol coated Raman-active-silica-gold-nanoparticles (PEG-R-Si-Au-NPs) in the liver of intrarectally administered or tail-vein injected mice. Tissue samples were trimmed to <1 mm^3 and prepared and stained by osmium tetroxide and uranyl acetate. Sections (150 nm thick) were cut from the block face using an ultramicrotome and then placed on a copper grid. This approach utilizes the simultaneous bright and dark field imaging capabilities of STEM to readily identify PEG-R-Si-Au-NPs in mouse liver tissue. Results showed that nanoparticles injected intravenously accumulated in the liver while those administered intrarectally did not, indicating that they remain in the colon and do not pass through the colon wall into the systemic circulation.

Overall, the main advantage with electron microscopy is the high resolution, which allows visualization of the accumulation of nanoparticles in cells and the localization of nanoparticles in cellular organelles (Jong et al., 2010). Although this technique is generally considered a semi-quantitative method, a number of limitations should be considered when approaching this technique for evaluating nanoparticle biodistribution. Electron microscopy is a more expensive technique and is not capable of evaluating large tissue sections compared to standard histology (**Table 1**). For example, the analysis volume for TEM is generally low at 1–10 μm^3 for a single TEM session (Kempen et al., 2013). In addition, a limited number of ultra-thin tissue sections (50–150 nm thickness) are typically chosen to evaluate and approximate biodistribution in each organ, which may affect the results as not all sections are examined. Therefore, appropriate sampling methods and additional analytical methods should be utilized to provide a more reliable representation of nanoparticle biodistribution in the whole organ. Electron microscopy is also a time-consuming technique, with individual samples usually taking >3–4 h to analyze (Kempen et al., 2013). Relatively high numbers of nanoparticles need to be administered to enable the detection of nanoparticles in organs by electron microscopy, especially for larger nanoparticles (Jong et al., 2010; Kempen et al., 2013). An additional identification technique (e.g., EDX detection of the composing elements or a specific marker for the administered nanoparticles) may also be necessary for a positive identification of the nanomaterial in tissues and cells (Jong et al., 2010). Although electron microscopy can readily image soft matter samples, characterization of soft materials can be affected by the high-voltage electron beams (Garcia-Negrete et al., 2015). Therefore, artifacts need to be carefully protected against when an image is acquired, as burn-in spots can form on the image (Kempen et al., 2013). With regards to radiation exposure, low levels of X-rays can be produced from the backscattered electrons impinging on samples in electron microscopes. However, these units are well-shielded and any X-rays generated internally should not penetrate outside the unit. Finally, the sample preparation method will not be suitable for all nanoparticles. As this process involves the use of lipid soluble solvents, nanoparticles composed of materials that are easily degraded by these solvents (e.g., liposomes, solid lipid nanoparticles and micelles) should use other biodistribution techniques.

LIQUID SCINTILLATION COUNTING (LSC)

Liquid scintillation counting (LSC) is a standard laboratory method to quantify the radioactivity of low energy radioisotopes—most commonly beta-emitting (β-emitting) and alpha-emitting (α-emitting) isotopes (Shigematsu et al., 1995; PerkinElmer, 2008). LSC analysis of samples requires a specific cocktail containing the aromatic organic solvent and scintillators (also referred to as "fluors") to absorb the radioisotopic energy and produce detectable light pulses, respectively. The basic principles of LSC rely on the energy released from a radioactive decay (emitting beta or alpha particles) to excite the aromatic solvent molecules. The energy of the solvent molecules is then transferred to the scintillator molecules to produce excited states of the electrons, which decay to the ground state and produce a light pulse that is characteristic for the scintillator. The emitted light is detected by the photomultiplier tube (PMT) of the liquid scintillation counter.

In vivo biodistribution of nanomedicines can be assessed by labeling nanoparticles with isotopic markers prior to administration in animals. For example, anti-ICAM-1 immunoliposomes and control liposomes were radiolabeled with [^{3}H]-CHE and administered intravenously in rats with Complete Freund's Adjuvant-induced inflammation of the paw (Hua and Cabot, 2013). The use of [^{3}H]-CHE is convenient for these studies because it is a stable, non-exchangeable, and non-degradable marker of liposomes, thus providing an estimate of the cumulative liposome dose in tissues (Hua and Cabot, 2013). Organs are then harvested and prepared for LSC. Depending on the sample type, the biological material can either

be directly mixed to the cocktail with no or little pre-treatment, or a treatment/solubilization may be needed prior to scintillation cocktail addition (PerkinElmer, 2008). The latter is generally required when analyzing biological tissues and usually a portion of an organ is weighed and processed, due to the time taken for effective solubilization of larger tissues. Radioactivity is measured in terms of number of disintegrations per minute (DPM) of the isotope in each tissue sample. The amount of radioactivity can then be expressed as the number of becquerel (Bq) per gram of tissue using the following conversion: 1 Bq = 60 DPM. The becquerel is the SI derived unit of radioactivity.

LSC has the advantages of being a sensitive, specific, and quantitative technique for measuring nanomedicine biodistribution (**Table 1**). Removal of excess free isotopic markers that have not been incorporated into the nanoparticles is important prior to *in vivo* assessment. It should be noted that the quality and reproducibility of the data will depend on the choice of the cocktail as well as on the sample composition, volume, temperature, and counting device (PerkinElmer, 2008). This technique can be laborious, especially with the need to treat and solubilize the harvested tissues prior to LSC analysis. If a small portion of an organ is collected for LSC, this may not be an accurate reflection of whole organ biodistribution. Furthermore, LSC can only determine nanoparticle biodistribution at the tissue or organ level and does not provide any information regarding specific cellular association or accumulation of nanoparticles in tissues.

MEASUREMENT OF DRUG CONCENTRATION IN TISSUES

Nanoparticles loaded with therapeutic compounds can have their biodistribution evaluated by measuring drug concentration in tissues. This is an indirect approach and more specifically determines payload biodistribution. The assumption is that nanoparticles accumulate in specific tissues following *in vivo* administration, where they then release their cargo. It does not take into account possible premature drug release from the nanoparticles into the circulation and subsequent biodistribution of the free drugs themselves. This technique involves tissue samples being prepared for solubilization and extraction of the specific compound for further analysis. In order to achieve effective drug extraction from tissues, it is important to first determine the physicochemical properties of the compound and tissue matrix in the sample (Pavlović et al., 2007).

In brief, biological tissues are broken down by methods such as grinding, blending, homogenization, sonication or sieving, as finer samples are more homogenous and easier to extract. Particulates are removed from the coarse biological material through methods such as centrifugation, filtration or solid-phase extraction. The supernatant is then collected and subjected to further extraction and purification. The extraction of drugs from biological tissues depends on its physicochemical properties, such as solubility, hydrophobicity/hydrophilicity, ionization, partition coefficient, and molecular weight. For example, solid-liquid extraction may be used, where a solvent is added to dissolve the analyte in the sample. The mixture is then filtered, decanted, or centrifuged to separate the solvent from the remaining sample. Following extraction, evaporation and reconstitution may be required before final analysis with high-performance liquid chromatography (HPLC) and/or mass spectrometry (MS) (Majors, 2013).

Measurement of drug concentration in tissues has been widely used for determining the biodistribution of nanomedicines. For example, Milane et al. (2011) assessed the biodistribution of epidermal growth factor receptor (EGFR)-targeted polymer-blend nanoparticles loaded with the anti-cancer drugs, lonidamine and paclitaxel, in an orthotopic animal model of multi-drug resistant breast cancer. After euthanasia, the tumor mass, liver, lungs, kidneys, spleen, and heart were harvested and weighed. Tissue and plasma samples were then prepared using established methods for the extraction of lonidamine and paclitaxel in preparation for HPLC analysis. The data showed that both the non-targeted and the targeted nanoparticles were effective at increasing the tumor concentration of paclitaxel and lonidamine relative to free drug solution.

The main advantage of this technique is that it provides a quantitative measure of biodistribution that can be used to analyze whole or partial tissue samples (**Table 1**). This method does not involve exposure to ionizing radiation, incorporation of imaging molecules to nanoparticles, or the administration of contrast agents to enhance imaging outcomes. However, as mentioned earlier this indirect technique more specifically measures payload biodistribution. It is the compound encapsulated into or incorporated on the surface of the nanoparticles that is measured, which may provide unreliable results if the compound prematurely dissociates from the nanoparticles following *in vivo* administration. Furthermore, accurate measurement of drug concentration is highly dependent upon the quality of the tissue preparation and extraction procedure, which can be time-consuming and laborious. This technique is also unable to provide information on real-time biodistribution across time points in animals, but can be used as a secondary quantitative measure to support the biodistribution results attained from qualitative techniques.

IN VIVO OPTICAL IMAGING

This technique refers to the use of equipment such as the *In Vivo* Imaging System (IVIS®) and Kodak *In-Vivo* FX Imaging Station to visualize the biodistribution of nanoparticles in real-time in live animals or in harvested tissues and organs. These *in vivo* imaging systems are non-invasive and involve optical imaging technology to evaluate fluorescence or bioluminescence within the sample. Even though *in vivo* imaging systems typically possess these dual imaging capabilities, fluorescence imaging is used the most to evaluate the biodistribution of nanoparticles. Fluorescent imaging employs the ability of fluorophores, such as fluorescent proteins, dyes and conjugated polymers, to fluoresce after being excited with light of a particular wavelength (Janib et al., 2010; Coll, 2011; Priem et al., 2015). Fluorophores can be encapsulated within the nanoparticles (core or membrane)

or conjugated to the nanoparticle surface. To optimize *in vivo* imaging sensitivity, fluorescent contrast agents should emit light in the red or near infrared (near-IR) wavelengths (∼600–1,000 nm) (Coll, 2011; Liu Y. et al., 2012). This is particularly important for deep tissue samples to avoid coinciding with low photon absorption and autofluorescence in tissues, thereby enabling higher signal-to-background ratios (Vats et al., 2017). Once the sample is excited by a light source within the imaging chamber, fluorescence is emitted and captured on a charge-coupled device (CCD) camera that then converts this into electrical signals (Coll, 2011). A three-dimensional, tomographic image depicting the biodistribution of the fluorescent probe is then reconstructured.

In vivo imaging systems are commonly used to evaluate the biodistribution of nanoparticles, particularly in live animals across various time points to assess accumulation relative to disease progression. A variety of fluorescent-labeled nanoparticles have been imaged using this technique, including nanoporous silicon nanoparticles, carbon nanotubes, metal-based nanoparticles, polymer-based nanoparticles, and lipid-based nanoparticles (Connell et al., 2002; Zheng et al., 2003; Gao et al., 2010b; Goldberg et al., 2011; Milane et al., 2011; Tasciotti et al., 2011; Liu Y. et al., 2012; Zhang et al., 2012). For example, Milane et al. (2011) used this technique as a qualitative assessment of the biodistribution of EGFR-targeted polymer-blend nanoparticles in an orthotopic animal model of multi-drug resistant breast cancer. In this study, non-targeted and targeted nanoparticles loaded with DiR (near-IR) dye were administered via tail vein injection, and the biodistribution was visualized using a Kodak *In-Vivo* FX Imaging Station over 6 h. The results attained from *in vivo* optical imaging were found to be comparable with the quantitative data attained from HPLC analysis of drug distribution. Interestingly, some nanoparticles possess contrast that is inherently fluorescent such as quantum dot nanocrystals (Gao et al., 2010a,b; Liu Y. et al., 2012; Zhang et al., 2012; Zhao and Zeng, 2015). Quantum dots are semiconductor nanocrystals synthesized with a core-shell structure that enables imaging in the near infrared spectrum, thereby enhancing image sensitivity. They possess attractive optical qualities such as size-tunable fluorescence, photostability, high fluorescence quantum yields, and high resistance to photobleaching (Gao et al., 2010a). However, quantum dot preparations contain heavy metals such as cadmium, tellurium and selenium, which are potentially toxic to the body (Hardman, 2006; Kim et al., 2017).

Overall, *in vivo* optical imaging has the advantages of being direct, non-invasive and relatively simple to conduct (**Table 1**). It has fast image acquisition times and the procedure does not require exposure to ionizing radiation (Koo et al., 2006; Liu Y. et al., 2012). As imaging can be performed in real-time, biodistribution of nanoparticles can be assessed over many time points in the same group of animals—thus allowing a reduction in animal numbers. The images produced tend to have high sensitivity and enhanced temporal resolution (Liu Y. et al., 2012; Kim et al., 2017). This technique is generally considered a qualitative measure of biodistribution, as the intensity measured is not necessarily relative to the number of nanoparticles present in the tissues (Liu Y. et al., 2012). There are also a few limitations to this technique that should be considered. *In vivo* imaging systems can have limited tissue penetration (<1 cm) and is prone to attenuation with increased tissue depth (Koo et al., 2006; Kim et al., 2017). This is due to interference from light absorption and light scattering by tissue biomatter. This technique also has relatively low spatial resolution compared to CT and MRI (Massoud and Gambhir, 2003). In addition, labeling of nanoparticles with fluorophores may alter their physicochemical properties (e.g., surface charge, size, and surface functionalization) and *in vivo* behavior (Ann et al., 2013; Robson et al., 2018). Therefore, the choice of fluorophore and the method for labeling nanoparticles should be carefully considered. Another concern is that many fluorophores can undergo photobleaching (Robson et al., 2018), which affects their sensitivity to imaging. Tissue autofluorescence is a significant issue that can affect the interpretation of results, therefore fluorophores should have high signal-to-background ratios (Koo et al., 2006). Furthermore, *in vivo* imaging systems can only determine nanoparticle biodistribution at the tissue or organ level and do not provide any information regarding specific cellular association or accumulation of nanoparticles in tissues. It cannot visualize individual nanoparticles, but instead measures broader fluorescence intensity.

COMPUTED TOMOGRAPHY (CT)

Computed tomography (CT) is a non-invasive, radiological imaging technique that uses X-rays to produce three-dimensional, tomographic (cross-sectional) images of tissues. This technique is based on the variable absorption of X-rays by different tissues, which is a form of ionizing radiation with wavelengths of ∼0.01–10 nm (Kim et al., 2017). CT scanners typically consist of an X-ray tube, a detector unit, an image reconstruction system, collimators and filters. The X-ray tube is composed of a cathode and a tungsten-alloy anode housed within a vacuum. X-rays are generated within the tube by applying high voltage, which accelerates electrons from the heated cathode filament toward the anode. The accelerated electrons interact with electrons of the anode's tungsten nuclei and subsequently cause emission of X-rays. X-rays are then passed through the subject and are attenuated (absorbed or scattered), resulting in a loss of X-ray intensity (Lusic and Grinstaff, 2013; Liguori et al., 2015; Kim et al., 2017). Differential attenuation of X-rays across tissues according to their attenuation coefficient causes variation in radiation intensities and depicts information about tissue density and structure (Chatterjee et al., 2014; Liguori et al., 2015). This information is captured by detectors as a series of projections. Usually, the X-ray tube and detectors rotate synchronously on a circular axis around the subject with detectors positioned directly opposite, which enables a complete dataset of projections to be obtained over 360°. Computer algorithms are then applied to produce a three-dimensional reconstruction of the scanned object. Collimators and filters are used to limit unwarranted radiation and enhance the quality of the image (Liguori et al., 2015).

Contrast within the final image depends on the different densities and thickness of body structures. While different types of tissues can exhibit contrast, it can be particularly challenging to achieve high quality images and identify the interface between two different adjacent tissues (e.g., tumor in an organ) or to image soft tissues in contact with bodily fluids (Lusic and Grinstaff, 2013; Chatterjee et al., 2014). Therefore, contrast imaging agents are often used to increase CT sensitivity to enhance visualization and differentiation among different tissues. Contrast agents are usually elements having high atomic numbers and, therefore, higher number of electrons, which attenuate X-rays more efficiently by absorbing external X-rays. This results in decreased exposure on the X-ray detector (Lusic and Grinstaff, 2013). Contrast agents used clinically in patients undergoing CT are typically iodine- or barium-based compounds. Iodinated contrast agents are the main type of radiocontrast used for vascular imaging (e.g., vascular calcifications and hemorrhage), whereas barium sulfate is mainly used for imaging the gastrointestinal tract (Lusic and Grinstaff, 2013; Chatterjee et al., 2014; Kim et al., 2017).

CT has been utilized as a technique to allow *in vivo* imaging of the biodistribution of nanoparticles in real-time. Electron-dense elements are typically incorporated into the nanoparticles to enable visualization and differentiation of the nanoparticles in the tissues. Contrast agents that are more commonly incorporated into nanoparticles for CT analysis include iodine (Torchilin et al., 1999; Yordanov et al., 2002; Fu et al., 2006; Ho Kong et al., 2007; Elrod et al., 2009; de Vries et al., 2010; Hill et al., 2010; Hallouard et al., 2011), gold (Chie et al., 2010; Guo et al., 2010; Wang et al., 2011; Xiao et al., 2013), and bismuth (Rabin et al., 2006; Naha et al., 2014). However, various other elements such as gadolinium (Zhou et al., 2014), platinum (Chou et al., 2010), tantalum (Bonitatibus et al., 2010; Oh et al., 2011), tungsten (Jakhmola et al., 2014; Firouzi et al., 2017), and ytterbium (Pan et al., 2012; Jianhua et al., 2013) have also been used. Contrast agents for CT imaging can be loaded into the core of the nanoparticles, chemically grafted to the surface of nanoparticles, or inserted into the carrier membrane (e.g., lipid bilayer) (Cormode et al., 2014; Li et al., 2014). The *in vivo* biodistribution of numerous types of nanoparticles have been studied with CT, including nano-emulsions (de Vries et al., 2010; Hallouard et al., 2011), liposomes (Sachse et al., 1997; Leander et al., 2001; Elrod et al., 2009), micelles (Torchilin et al., 1999; Torchilin, 2002), lipoproteins (Cormode et al., 2008; Hill et al., 2010), polymer-coated nanoparticles (Rabin et al., 2006; Muddineti et al., 2015; Firouzi et al., 2017), nanocapsules/nanospheres (Ashcroft et al., 2007; Ho Kong et al., 2007), nanotubes/nanorods (Ashcroft et al., 2007; Zhou et al., 2014), metal-based nanoparticles (Bonitatibus et al., 2010; Chou et al., 2010; Oh et al., 2011; Pan et al., 2012; Jianhua et al., 2013; Mieszawska et al., 2013; Cormode et al., 2014; Jakhmola et al., 2014; Naha et al., 2014; Kim et al., 2017), and dendrimers (Yordanov et al., 2002; Fu et al., 2006; Chie et al., 2010; Guo et al., 2010; Wang et al., 2011; Xiao et al., 2013).

CT has demonstrated to be an effective technique for producing reliable and high-resolution images for assessing the biodistribution of nanoparticles (**Table 1**). It has no tissue penetration limits and relatively quick image acquisition times (Massoud and Gambhir, 2003). This technique is generally considered a qualitative measure of biodistribution and can only determine nanoparticle biodistribution at the tissue or organ level. Furthermore, CT requires exposure to ionizing radiation and does not provide any information regarding specific cellular association of nanoparticles (Kim et al., 2017). Biodistribution of nanoparticles can be assessed in real-time and over many time points in the same group of animals, which reduces the number of animals required for longitudinal studies. However, CT alone can suffer from relatively poor visualization and differentiation among different types of tissues as mentioned above (Lusic and Grinstaff, 2013; Chatterjee et al., 2014). Hence, it often requires the administration of contrast imaging agents to increase CT sensitivity. This can pose a problem when nanoparticles labeled with contrast agents are used in conjunction with other contrast imaging agents to improve anatomical and tissue imaging. The detection limit of nanoparticle contrast agents is less sensitive compared to other modalities, such as nuclear imaging (Massoud and Gambhir, 2003; Kim et al., 2017). To overcome this issue, nanoparticles incorporating high concentrations of contrast agents are often required to improve imaging. Incorporation of contrast agents in nanoparticles may alter their physicochemical properties and *in vivo* behavior (Massoud and Gambhir, 2003; Kim et al., 2017).

MAGNETIC RESONANCE IMAGING (MRI)

Magnetic resonance imaging (MRI) is a non-invasive imaging technique that produces three dimensional detailed anatomical images, without the use of ionizing radiation. MRI uses powerful magnets that produce a strong magnetic field that forces protons in the body to align with that field (Strijkers et al., 2007; Grover et al., 2015). Protons (hydrogen nuclei) are typically used in MRI imaging as they are particularly abundant in the water and fat of the body. Protons possess a positive charge and are constantly spinning around their own axes, which generates a magnetic field. The magnetic field for each proton is known as a magnetic moment and is a measure of an object's tendency to align with a magnetic field. Radiofrequency currents are pulsed through the patient to excite the protons to a higher energy state and spin them out of equilibrium, which creates strain against the pull of the magnetic field (Grover et al., 2015). When the radiofrequency field is turned off, the protons then realign with the magnetic field and the MRI sensors can detect the energy that is released in this process. In particular, MRI is able to produce high-resolution images by measuring the spin magnetization of polarized protons and their respective longitudinal (T_1) and transverse (T_2) relaxation rates in the body. It utilizes magnetic fields, electric field gradients and radio waves to produce three types of images: spin density weighted, T_1 weighted and T_2 weighted images (Strijkers et al., 2007; Grover et al., 2015). Field gradient coils are used to localize the MRI signal to particular tissues of interest. The signals are processed to extract frequency and phase data, and a mathematical algorithm is applied to construct an image. The

time it takes for the protons to realign with the magnetic field and the amount of energy released changes depending on the environment and the chemical nature of the molecules, which is used to differentiate between various types of tissues (Grover et al., 2015).

Contrast agents may be administered to a patient intravenously before or during the MRI procedure to increase the speed at which protons realign with the magnetic field, thereby shortening the T_1 and/or T_2 relaxation rates of protons located in their vicinity. Contrast agents that shorten T_1 (paramagnetic contrast agents) result in T_1 and T_1^* hypersignal (brighter images), whereas those that shorten T_2 (superparamagnetic contrast agents) lead to a reduction in the T_2 and T_2^* signal (darker images) (Strijkers et al., 2007; Kamaly and Miller, 2010). This improvement in image quality also enhances the differentiation between tissues. The effectiveness of contrast agents depends on its relaxivity, which is the proportionality constant of the measured rate of relaxation: $1/T_1$ and $1/T_2$ (Sun et al., 2008). Superparamagnetic iron oxide crystals (Fe^{3+} or Fe^{2+}) and paramagnetic lanthanide metals, such as gadolinium (Gd^{+3}), are the most widely used contrast agents for MRI imaging (Strijkers et al., 2007; Kamaly and Miller, 2010). Superparamagnetic iron oxide (SPIO, >50 nm in size) and ultrasmall superparamagnetic iron oxide (USPIO, <50 nm in size) are mainly used to shorten T_2, leading to darker images in T_2 and T_2^* weighted MRI (Jung and Jacobs, 1995; Strijkers et al., 2007; Kamaly and Miller, 2010). Conversely, paramagnetic gadolinium ions are used to shorten T_1, resulting in brighter images in T_1 weighted MRI (Strijkers et al., 2007; Kamaly and Miller, 2010). The most clinically used MRI contrast agents are those that shorten T_1 relaxation rates, hence those that contain the element gadolinium are often preferred (Sun et al., 2008).

Gadolinium (Gd^{+3}) has seven unpaired outer shell electrons and a large magnetic moment, making it extremely useful for MRI imaging (Strijkers et al., 2007; Kamaly and Miller, 2010). Free gadolinium ions are highly toxic and, therefore, they are usually chelated with other ligands (e.g., diethylenetriamine pentaacetic acid, DTPA; tetraazacyclododecane tetraacetic acid, DOTA) to form complexes that are nontoxic and highly stable in the body during the period of administration (Wiegers et al., 1992; Rosen et al., 2011). For example, Park et al conjugated the peptide RGD to Gd-DOTA to obtain an MRI contrast agent with tumor targeting capability (Park et al., 2008). One of the more common gadolinium chelate used clinically and in drug delivery is gadoteridol, which is the chelate formed between Gd^{+3} and 10-(2-hydroxy-propyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetate (Zhou and Lu, 2013). Gadolinium-based contrast agents can be incorporated into nanoparticles to enable real-time imaging of their *in vivo* biodistribution using MRI. A number of nanoparticle types have incorporated these contrast agents, including liposomes (Unger et al., 1989; Saito et al., 2005; Hossann et al., 2013; Smith et al., 2013; Skupin-Mrugalska et al., 2018), dendrimers (Margerum et al., 1997; Lee et al., 2005; Rongzuo et al., 2007), micelles (Parac-Vogt Tatjana et al., 2004; Kumar et al., 2010), polymeric-based nanoparticles (Liu et al., 2011), carbon-based nanotubes (Hartman et al., 2008; Richard et al., 2008), and mesoporous silica nanoparticles (Kobayashi et al., 2007; Kim et al., 2008; Taylor et al., 2008). For example, Saito et al. (2005) manufactured gadoteridol-loaded liposomes for real-time MRI evaluation of convection-enhanced delivery in the primate brain. Volume of distribution was analyzed for all delivery locations by histology and MRI, following administration in the corona radiata, putamen nucleus, and brain stem. The results showed that MRI of liposomal gadolinium was highly accurate at determining tissue distribution, as confirmed by comparison with histological results from concomitant administration of fluorescent liposomes. Gadolinium-based contrast agents can be incorporated within the core of nanoparticles, attached to the particle surface, or inserted into the carrier membrane (Unger et al., 1989; Hossann et al., 2013; Smith et al., 2013; Skupin-Mrugalska et al., 2018). It should be noted that encapsulation of gadolinium within the core can lead to lowered relaxivity, whereas surface attachment may be preferable to improve gadolinium's ability to interact with water (Tilcock et al., 1989; Kamaly and Miller, 2010). Relaxivity can be further improved by reducing the size of the nanoparticles (Tilcock et al., 1989; Kamaly and Miller, 2010).

MRI can also be used to evaluate the biodistribution of nanoparticles formulated with superparamagnetic iron oxide cores. Iron oxide crystals are mainly utilized to provide negative contrast in T_2 and T_2^* weighted images. SPIO and USPIO nanoparticles are usually composed of a nano-sized magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) core coated with a variety of materials to enhance stability, circulation time, biocompatibility and minimize toxicity (Peng et al., 2008). Bulk iron oxide is ferromagnetic, however when nano-sized, superparamagnetism is exhibited (Di Marco et al., 2007). The superparamagnetism of iron oxide nanoparticles is important for *in vivo* imaging. Polymers are the most widely used stabilizing materials and can be adsorbed into or anchored onto the iron oxide surface via hydrogen bonds, electrostatic forces or pseudo-covalent bonding (Estelrich et al., 2015). Examples include poly(ethylene glycol) (PEG), alginate, chitosan, dextran and its derivatives, starch, polyvinyl alcohol, albumin, poly(ethylene imine), organic siloxane, and sulphonated styrene-divinyl-benzene (Estelrich et al., 2015). In addition, SPIO can be used alone or incorporated into other nanostructures, such as magnetoliposomes (SPIOs are hybridized within a liposome carrier) (Martina et al., 2005; Plassat et al., 2007) and colloidal iron oxide nanoparticles (oleate-coated magnetite particles embedded in a hydrophobic matrix) (Senpan et al., 2009). Several formulations of iron oxide nanoparticles are already approved for clinical use (e.g., ferumoxides and ferucarbotran) for contrast-enhanced MRI of the liver (Reimer and Tombach, 1998). Their relatively large surface area also enables incorporation of biologically active substances to the surface of the nanoparticles. For example, Veiseh et al. (2009) developed a nanoprobe consisting of an iron oxide nanoparticle coated with biocompatible PEG–grafted chitosan copolymer, which allowed conjugation of a tumor-targeting agent, chlorotoxin, and a near-IR fluorophore. The results showed an ability for the nanoprobe to cross

the blood-brain barrier and specifically target brain tumors in a genetically engineered mouse model, as evidenced by *in vivo* MRI evaluation, *in vivo* optimal imaging and histology. The magnetism and subsequent MRI effectiveness of iron oxide nanoparticles is dependent upon their size, shape, morphology, structure, and homogeneity (Lin et al., 2012; Estelrich et al., 2015). Thus, variations in SPIO and USPIO nanoparticles can lead to different magnetic properties and thus alter their function in various applications. The coating and surface modifications can also influence *in vivo* stability and biodistribution of the nanoparticles. It should be noted that for conventional MRI, SPIO nanoparticles give negative contrast enhancement (dark signals) that are often confounded by the presence of artifacts due to hemorrhage, air, and partial-volume effects. To address these issues, many attempts have been made to generate positive contrast visualization methods in the last decade (Lin et al., 2012; Estelrich et al., 2015).

Overall, MRI has the advantage of producing high spatial resolution images (micrometers rather than several millimeters) compared to other techniques such as optical or radionuclide imaging (Massoud and Gambhir, 2003) (**Table 1**). It provides better soft tissue contrast than CT and can differentiate better between fat, water, muscle, and soft tissue (Massoud and Gambhir, 2003; Janib et al., 2010). MRI is not limited by tissue depth (unlimited penetration) and does not involve exposure to ionizing radiation. Furthermore, this technique allows non-invasive, three-dimensional, real-time imaging of the biodistribution of nanoparticles *in vivo*. However, MRI is more costly and has slow image acquisition and long post-processing times (Kim et al., 2017). As this technique can suffer from poor sensitivity, much higher amounts of contrast agent generally need to be administered (Massoud and Gambhir, 2003; Kim et al., 2017). MRI cannot be used in subjects with metallic implants/devices (Janib et al., 2010). In addition, incorporation of contrast agents in nanoparticles may alter their physicochemical properties and *in vivo* behavior (Massoud and Gambhir, 2003; Kim et al., 2017).

NUCLEAR MEDICINE IMAGING

Single photon emission computed tomography (SPECT) and positron emission tomography (PET) scans are the two most common imaging modalities in nuclear medicine. They are both non-invasive techniques that produce three-dimensional images of the body by detecting gamma rays (γ-rays) that are emitted from radioactive substances that become localized and are taken up by specific tissues (Townsend, 2004; Ziegler, 2005; Pimlott and Sutherland, 2011; Van Audenhaege et al., 2015). Both techniques essentially involve administration of a radioactive tracer (radiotracer) into the subject that consists of a molecular probe with a radioactive isotope attached that is capable of emitting γ-rays. The choice of molecular probe is dependent on the tissue to be imaged and should ideally have high affinity and high selectivity for the target receptor or organ (Pimlott and Sutherland, 2011). As the isotope decays in the tissue, it emits gamma rays that are picked up by detectors (gamma scintillation camera system) placed around the subject. The scintillation crystals within the detectors then convert the γ-ray energy into lower-energy (near-optical) photons. This optical energy is converted into electrical signals by photomultiplier tubes and processed to obtain the location of the scintillation events in the crystal (Townsend, 2004; Ziegler, 2005; Peterson and Furenlid, 2011). The radionuclide is captured in a collection of projections, which are measured from numerous angles and linear positions in the subject. Image reconstruction techniques are applied to reconstruct these projections into a three-dimensional, tomographic image of the radiotracer's biodistribution and concentration within the tissue (Townsend, 2004; Ziegler, 2005; Peterson and Furenlid, 2011; Pimlott and Sutherland, 2011).

Although both PET and SPECT rely on the detection of gamma radiation, they differ in the type of radionuclides used. The radionuclides used in SPECT emit γ-rays by radioactive decay that is measured directly, whereas PET radionuclides emit positrons that annihilate with electrons up to a few millimeters away in the tissue to produce two gamma photons that are emitted in opposite directions (Massoud and Gambhir, 2003; Townsend, 2004; Ziegler, 2005). The γ-rays emitted in PET are captured in coincidence by opposing pairs of detectors aligned collinearly around the subject, which enable measurement of the radionuclide from multiple angles and planes (Townsend, 2004; Ziegler, 2005). Unlike PET, SPECT gamma cameras are rotated around the subject and a lead collimator is required to reconstruct the original location of the emitted γ-rays (Peterson and Furenlid, 2011; Van Audenhaege et al., 2015). PET positron emitters (e.g., ^{15}O, ^{64}Cu, ^{13}N, ^{11}C, and ^{18}F) emit higher energy γ-rays and possess shorter radioactive half-lives than SPECT radiotracers (Massoud and Gambhir, 2003; Townsend, 2004; Ziegler, 2005). The most common radioisotopes used for SPECT imaging include ^{99m}Tc, ^{111}In, and radioiodine (e.g., ^{131}I) (Hong et al., 2009; Pimlott and Sutherland, 2011).

Nanoparticles can be labeled with gamma-emitting radionuclides and positron emitters. These radiolabels can be attached to the nanoparticle surface, conjugated to the nanoparticle core, or encapsulated within a payload that is loaded into the nanoparticle. Radiolabeling is achieved through methods such as exogenous chelation of radiometals, direct proton/neutron bombardment, and chelator-free radiolabeling (Gibson et al., 2011; Liu T. et al., 2012 Sun et al., 2015; Lu et al., 2018; Yuan et al., 2018). Alternatively, radioactive precursors can be used to synthesize intrinsically radioactive nanoparticles (Zhao et al., 2014; Sun et al., 2015). The type of radionuclide and radiolabeling strategy requires careful consideration, as some nanoparticles may exhibit differing compatibility and imaging effectiveness across the various methods (Liu and Welch, 2012). PET can be used to image the biodistribution of a variety of nanoparticles, including quantum dots (Ducongé et al., 2008; Tu et al., 2011), iron oxide nanoparticles (Glaus et al., 2010; Yang et al., 2011), gold nanoparticles (Xie et al., 2010; Guerrero et al., 2012), liposomes (Oku et al., 2011; Petersen et al., 2011), solid lipid nanoparticles (Andreozzi et al., 2011), polymer-based nanoparticles (Fukukawa et al.,

2008; Herth et al., 2009; Allmeroth et al., 2013), carbon-based nanoparticles (Liu et al., 2006; McDevitt et al., 2007), and micelles (Xiao et al., 2012). Similarly, SPECT imaging, often in combination with other imaging modalities, can also image the biodistribution of a similar range of nanoparticles, including dendrimers (Zhang et al., 2010a,b), micelles (Cheng et al., 2013; Hong et al., 2014), liposomes (Chang et al., 2010), carbon-based nanoparticles (Wu et al., 2009), iron-oxide nanoparticles (Madru et al., 2012), polymeric nanoparticles (Lu et al., 2011), gold nanoparticles (Morales-Avila et al., 2011; You et al., 2012), and silver nanoparticles (Chrastina and Schnitzer, 2010).

As PET and SPECT imaging rely purely on the detection of γ-rays, radiolabels must remain attached to the nanoparticles to accurately image their biodistribution. If disassociation occurs, imaging will not reflect true biodistribution, resulting in misleading and incorrect information (Liu and Welch, 2012). Therefore, it is important that the radiolabeling strategy, radionuclide type, and nanoparticle material are compatible, suited to the study purpose, and possess high *in vivo* stability (Liu and Welch, 2012; Sun et al., 2015). Although exogenous chelation of radionuclides is relatively easy, efficient and low cost, the resulting stability of radiolabels can be potentially problematic. Radionuclides may detach from chelators through trans-chelation or chelators may interact *in vivo* and subsequently disassociate from the nanoparticle (Bass et al., 2000; Boswell et al., 2004; Sun et al., 2015). The attachment of a chelator may also influence or damage the surface properties of nanoparticles, as high temperatures are required for chelation (Lu et al., 2018). This can adversely affect the conjugation capacity of targeting ligands (e.g., antibodies and PEG density), thereby resulting in impaired targeting, reduced circulation times and decreased imaging activity (Chang et al., 2008; Moghimi et al., 2012; Lu et al., 2018). Chelation issues can be avoided with direct bombardment radiolabeling, however this technique is limited by high costs, complexity of use, and the potential to damage the nanoparticles with ion-beam/neutron irradiation (Gibson et al., 2011). While intrinsic radioactive nanoparticles can exhibit high stability with limited radiolabel detachment, potential long-term toxicity and its limited applicability to only a few radioisotope-nanoparticle combinations present challenges for this technique (Liu T. et al., 2012; Chen et al., 2013; Goel et al., 2014). Furthermore, the chelator-free post-synthetic radiolabeling approach is fast, specific and can produce a high labeling yield, however is again limited to only a few nanoparticle and isotope combinations (Chen et al., 2013; Sun et al., 2015).

In comparison to other imaging modalities, PET and SPECT have the advantages of being able to image biochemical processes and are highly sensitive (nanomolar to picomolar level) (**Table 1**). Therefore, signals can be detected with very small amounts of labels which minimizes the disruption of cell function and surrounding tissue (Townsend, 2004; Ziegler, 2005; Pimlott and Sutherland, 2011; Van Audenhaege et al., 2015). These nuclear medicine imaging techniques are also quantitative and not restricted by tissue penetration limits (Koo et al., 2006; Janib et al., 2010; Kim et al., 2017). Several limitations should be considered for PET and SPECT imaging, including exposure to ionizing radiation, high costs, slow image acquisition times, and inability to be used for longitudinal studies due to radiolabel decay (Kim et al., 2017). Both imaging techniques also have low spatial resolution and provide a lack of anatomic information, hence they are often combined with other imaging modalities such as MRI or CT (Janib et al., 2010; Kim et al., 2017). When comparing between the two imaging techniques, SPECT has low photon detection efficiency and relatively low resolution due to the use of collimation, whereas PET is much more sensitive and provides more radiation event localization data owing to the detection of emissions "coincident" in time (Koo et al., 2006). The positron-emitting isotopes used in PET are also able to be substituted for naturally occurring atoms, thereby enhancing the ability to image molecular events (Massoud and Gambhir, 2003). However, SPECT can image multiple radionuclide probes simultaneously and is more widely available. SPECT scans are also significantly less expensive than PET scans, partly because their radionuclides are simple to prepare, more easily obtained, and typically possess longer half-lives than PET radionuclides (Massoud and Gambhir, 2003; Janib et al., 2010; Pimlott and Sutherland, 2011). In addition, PET typically requires a cyclotron or generator (Massoud and Gambhir, 2003).

CONCLUSION

There is a range of techniques available for evaluating the biodistribution of nanoparticles *in vivo*. In general, the choice of technique depends on the: (i) physicochemical characteristics of the nanoparticle formulation; (ii) compatibility and stability of the nanoparticles with different labels and labeling methods; (iii) study duration (single or multiple time points); (iv) analysis type (quantitative or qualitative); (v) sample type (whole animal, whole organ/tissue, or tissue sections); and (vi) degree of detail required (organ/tissue accumulation or cellular association). Other aspects that should be considered include accessibility, costs, accuracy, image resolution, toxicity, complexity, and duration of the procedure. Each technique has its own advantages and limitations, as well as capabilities for assessing real-time, whole-organ, and cellular accumulation. The techniques which allow real-time and qualitative imaging of biodistribution in live animals are *in vivo* optical imaging, CT, MRI and nuclear medicine imaging (PET and SPECT). PET and SPECT are also able to provide quantitative data of uptake into specific organs or tissues, along with LSC and indirectly measuring drug concentration. Of the techniques available, only *in vivo* optimal imaging, CT and MRI are capable of imaging nanoparticle biodistribution across multiple time-points in longitudinal studies. In addition, histology and electron microscopy are the only techniques that can provide detailed information on the cellular association of nanoparticles following *in vivo* administration. Research on the use of other modalities for studying the biodistribution of nanoparticles *in vivo* are currently being explored, including ultrasound imaging of nanoparticles loaded with ultrasound contrast agents (e.g., insoluble gas

perfluorocarbons or sulfur hexafluoride) (Janib et al., 2010; Shapiro et al., 2014; Kim et al., 2017). Nanoparticles with multifunctional theranostic capabilities, incorporating multi-mode contrast agents are also rapidly gaining popularity for biomedical applications.

AUTHOR CONTRIBUTIONS

SH: conception of the work. LA and SH: drafting of the manuscript. SH: preparation of the figure and table. SH, DS, JF, WP, AM, and AW: reviewing the article critically for important intellectual content.

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REFERENCES

Allmeroth, M., Moderegger, D., Gündel, D., Buchholz, H.-G., Mohr, N., Koynov, K., et al. (2013). PEGylation of HPMA-based block copolymers enhances tumor accumulation *in vivo*: a quantitative study using radiolabeling and positron emission tomography. *J. Control. Release* 172, 77–85. doi: 10.1016/j.jconrel.2013.07.027

Alturkistani, H. A., Tashkandi, F. M., and Mohammedsaleh, Z. M. (2016). Histological stains: a literature review and case study. *Glob. J. Health Sci.* 8, 72–79. doi: 10.5539/gjhs.v8n3p72

Andreozzi, E., Seo, J. W., Ferrara, K., and Louie, A. (2011). A Novel method to label Solid Lipid Nanoparticles (SLNs) with (64)Cu for Positron Emission Tomography (PET) imaging. *Bioconjug. Chem.* 22, 808–818. doi: 10.1021/bc100478k

Ann, F. H., Linda, M. S., Dale, W. P., Tina, M. S., Bean, T. C., David, G. F., et al. (2013). Nanotechnology: toxicologic pathology. *Toxicol. Pathol.* 41, 395–409. doi: 10.1177/0192623312467403

Asem, H., Zhao, Y., Ye, F., Barrefelt, Å., Abedi-Valugerdi, M., El-Sayed, R., et al. (2016). Biodistribution of biodegradable polymeric nano-carriers loaded with busulphan and designed for multimodal imaging. *J. Nanobiotechnol.* 14:82. doi: 10.1186/s12951-016-0239-0

Ashcroft, J. M., Hartman, K. B., Kissell, K. R., Mackeyev, Y., Pheasant, S., Young, S., et al. (2007). Single-molecule I2@US-tube nanocapsules: a new X-ray contrast-agent design. *Adv. Mater.* 19, 573–576. doi: 10.1002/adma.200601424

Bass, L. A., Wang, M., Welch, M. J., and Anderson, C. J. (2000). *In vivo* transchelation of Copper-64 from TETA-octreotide to superoxide dismutase in rat liver. *Bioconjug. Chem.* 11, 527–532. doi: 10.1021/bc990167l

Bharali, D. J., and Mousa, S. A. (2010). Emerging nanomedicines for early cancer detection and improved treatment: current perspective and future promise. *Pharmacol. Ther.* 128, 324–335. doi: 10.1016/j.pharmthera.2010.07.007

Bonitatibus, P. J. Jr., Torres, A. S., Goddard, G. D., FitzGerald, P. F., and Kulkarni, A. M. (2010). Synthesis, characterization, and computed tomography imaging of a tantalum oxide nanoparticle imaging agent. *Chem. Commun.* 46, 8956–8958. doi: 10.1039/c0cc03302b

Boswell, C. A., Sun, X., Niu, W., Weisman, G. R., Wong, E. H., Rheingold, A. L., et al. (2004). Comparative *in vivo* stability of Copper-64-Labeled cross-bridged and conventional tetraazamacrocyclic complexes. *J. Med. Chem.* 47, 1465–1474. doi: 10.1021/jm030383m

Brown, A., and Hondow, N. (2013). Electron microscopy of nanoparticles in cells. *Front. Nanosci.* 5:95–120. doi: 10.1016/B978-0-08-098338-7.00004-2

Chang, M.-Y., Seideman, J., and Sofou, S. (2008). Enhanced loading efficiency and retention of 225Ac in rigid liposomes for potential targeted therapy of micrometastases. *Bioconjug. Chem.* 19, 1274–1282. doi: 10.1021/bc700440a

Chang, Y.-J., Chang, C.-H., Yu, C.-Y., Chang, T.-J., Chen, L.-C., Chen, M.-H., et al. (2010). Therapeutic efficacy and microSPECT/CT imaging of 188Re-DXR-liposome in a C26 murine colon carcinoma solid tumor model. *Nucl. Med. Biol.* 37, 95–104. doi: 10.1016/j.nucmedbio.2009.08.006

Chatterjee, K., Sarkar, S., Jagajjanani Rao, K., and Paria, S. (2014). Core/shell nanoparticles in biomedical applications. *Adv. Colloid Interface Sci.* 209, 8–39. doi: 10.1016/j.cis.2013.12.008

Chen, F., Ellison, P. A., Lewis, C. M., Hong, H., Zhang, Y., Shi, S., et al. (2013). Chelator-free synthesis of a dual-modality PET/MRI agent. *Angewand. Chem. Int. Edn.* 52, 13319–13323. doi: 10.1002/anie.201306306

Cheng, C.-C., Huang, C.-F., Ho, A.-S., Peng, C.-L., Chang, C.-C., Mai, F.-D., et al. (2013). Novel targeted nuclear imaging agent for gastric cancer diagnosis: glucose-regulated protein 78 binding peptide-guided (111)In-Labeled polymeric micelles. *Int. J. Nanomedicine* 8:1385–1391. doi: 10.2147/IJN.S42003

Chie, K., Yasuhito, U., Mikako, O., Atsushi, H., Yasuhiro, M., and Kenji, K. (2010). X-ray computed tomography contrast agents prepared by seeded growth of gold nanoparticles in PEGylated dendrimer. *Nanotechnology* 21:245104. doi: 10.1088/0957-4484/21/24/245104

Cho, M., Cho, W.-S., Choi, M., Kim, S. J., Han, B. S., Kim, S. H., et al. (2009). The impact of size on tissue distribution and elimination by single intravenous injection of silica nanoparticles. *Toxicol. Lett.* 189, 177–183. doi: 10.1016/j.toxlet.2009.04.017

Chou, S.-W., Shau, Y.-H., Wu, P.-C., Yang, Y.-S., Shieh, D.-B., and Chen, C.-C. (2010). *In vitro* and *in vivo* studies of FePt nanoparticles for dual modal CT/MRI molecular imaging. *J. Am. Chem. Soc.* 132, 13270–13278. doi: 10.1021/ja1035013

Chrastina, A., and Schnitzer, J. E. (2010). Iodine-125 radiolabeling of silver nanoparticles for *in vivo* SPECT imaging. *Int. J. Nanomedicine* 5, 653–659. doi: 10.2147/IJN.S11677

Coll, J.-L. (2011). Cancer optical imaging using fluorescent nanoparticles. *Nanomedicine* 6, 7–10. doi: 10.2217/nnm.10.144

Connell, M. J., Bachilo, S. M., Huffman, C. B., Moore, V. C., Strano, M. S., Haroz, E. H., et al. (2002). Band gap fluorescence from individual single-walled carbon nanotubes. *Science* 297, 593. doi: 10.1126/science.1072631

Cormode, D. P., Naha, P. C., and Fayad, Z. A. (2014). Nanoparticle Contrast Agents for computed tomography: a focus on micelles. *Contrast Media Mol. Imaging* 9, 37–52. doi: 10.1002/cmmi.1551

Cormode, D. P., Skajaa, T., van Schooneveld, M. M., Koole, R., Jarzyna, P., Lobatto, M. E., et al. (2008). Nanocrystal core high-density lipoproteins: a multimodality contrast agent platform. *Nano Lett.* 8, 3715–3723. doi: 10.1021/nl801958b

de Vries, A., Custers, E., Lub, J., van den Bosch, S., Nicolay, K., and Grüll, H. (2010). Block-copolymer-stabilized iodinated emulsions for use as CT contrast agents. *Biomaterials* 31, 6537–6544. doi: 10.1016/j.biomaterials.2010.04.056

Di Marco, M., Sadun, C., Port, M., Guilbert, I., Couvreur, P., and Dubernet, C. (2007). Physicochemical characterization of ultrasmall superparamagnetic iron oxide particles (USPIO) for biomedical application as MRI contrast agents. *Int. J. Nanomedicine* 2, 609–622.

Ducongé, F., Pons, T., Pestourie, C., Hérin, L., Thézé, B., Gombert, K., et al. (2008). Fluorine-18-Labeled phospholipid quantum dot micelles for *in vivo* multimodal imaging from whole body to cellular scales. *Bioconjug. Chem.* 19, 1921–1926. doi: 10.1021/bc800179j

Elrod, D. B., Partha, R., Danila, D., Casscells, S. W., and Conyers, J. L. (2009). An iodinated liposomal computed tomographic contrast agent prepared from a diiodophosphatidylcholine lipid. *Nanomedicine* 5, 42–45. doi: 10.1016/j.nano.2008.06.007

Estelrich, J., Sánchez-Martín, M. J., and Busquets, M. A. (2015). Nanoparticles in magnetic resonance imaging: from simple to dual contrast agents. *Int. J. Nanomedicine* 10:1727–1741. doi: 10.2147/IJN.S76501

Firouzi, M., Poursalehi, R., Delavari, H. H., Saba, F., and Oghabian, M. A. (2017). Chitosan coated tungsten trioxide nanoparticles as a contrast agent for X-ray computed tomography. *Int. J. Biol. Macromol.* 98, 479–485. doi: 10.1016/j.ijbiomac.2017.01.138

Fu, Y., Nitecki, D. E., Maltby, D., Simon, G. H., Berejnoi, K., Raatschen, H.-J., et al. (2006). Dendritic iodinated contrast agents with peg-cores for ct imaging: synthesis and preliminary characterization. *Bioconjug. Chem.* 17, 1043–1056. doi: 10.1021/bc060019c

Fukukawa, K.-I, Rossin, R., Hagooly, A., Pressly, E. D., Hunt, J. N., Messmore, B. W., et al. (2008). Synthesis and characterization of core–shell star copolymers for *in vivo* PET imaging applications. *Biomacromolecules* 9, 1329–1339. doi: 10.1021/bm7014152

Gao, J., Chen, K., Xie, R., Xie, J., Lee, S., Cheng, Z., et al. (2010a). Ultrasmall near-infrared non-cadmium quantum dots for *in vivo* tumor imaging. *Small* 6, 256–261. doi: 10.1002/smll.200901672

Gao, J., Chen, K., Xie, R., Xie, J., Yan, Y., Cheng, Z., et al. (2010b). *In vivo* tumor-targeted fluorescence imaging using near-infrared non-cadmium quantum dots. *Bioconjug. Chem.* 21, 604–609. doi: 10.1021/bc900323v

Garcia-Negrete, C. A., Jimenez de Haro, M. C., Blasco, J., Soto, M., and Fernandez, A. (2015). STEM-in-SEM high resolution imaging of gold nanoparticles and bivalve tissues in bioaccumulation experiments. *Analyst* 140, 3082–3089. doi: 10.1039/C4AN01643B

Gibson, N., Holzwarth, U., Abbas, K., Simonelli, F., Kozempel, J., Cydzik, I., et al. (2011). Radiolabelling of engineered nanoparticles for *in vitro* and *in vivo* tracing applications using cyclotron accelerators. *Arch. Toxicol.* 85, 751–773. doi: 10.1007/s00204-011-0701-6

Glaus, C., Rossin, R., Welch, M. J., and Bao, G. (2010). *In vivo* evaluation of 64Cu-Labeled magnetic nanoparticles as a dual-modality PET/MR imaging agent. *Bioconjug. Chem.* 21, 715–722. doi: 10.1021/bc900511j

Goel, S., Chen, F., Ehlerding, E. B., and Cai, W. (2014). Intrinsically radiolabeled nanoparticles: an emerging paradigm. *Small* 10, 3825–3830. doi: 10.1002/smll.201401048

Goldberg, M. S., Xing, D., Ren, Y., Orsulic, S., Bhatia, S. N., and Sharp, P. A. (2011). Nanoparticle-mediated delivery of siRNA targeting Parp1 extends survival of mice bearing tumors derived from Brca1-deficient ovarian cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 745–750. doi: 10.1073/pnas.1016538108

Goldstein, A., Soroka, Y., Frusic-Zlotkin, M., Popov, I., and Kohen, R. (2014). High resolution SEM imaging of gold nanoparticles in cells and tissues. *J. Microsc.* 256, 237–247. doi: 10.1111/jmi.12179

Grover, V. P. B., Tognarelli, J. M., Crossey, M. M. E., Cox, I. J., Taylor-Robinson, S. D., and McPhail, M. J. W. (2015). Magnetic resonance imaging: principles and techniques: lessons for clinicians. *J. Clin. Exp. Hepatol.* 5, 246–255. doi: 10.1016/j.jceh.2015.08.001

Guerrero, S., Herance, J. R., Rojas, S., Mena, J. F., Gispert, J. D., Acosta, G. A., et al. (2012). Synthesis and *in vivo* evaluation of the biodistribution of a 18F-Labeled conjugate gold-nanoparticle-peptide with potential biomedical application. *Bioconjug. Chem.* 23, 399–408. doi: 10.1021/bc200362a

Guo, R., Wang, H., Peng, C., Shen, M., Pan, M., Cao, X., et al. (2010). X-ray attenuation property of dendrimer-entrapped gold nanoparticles. *J. Phys. Chem. C* 114, 50–56. doi: 10.1021/jp9078986

Hallouard, F., Anton, N., Zuber, G., Choquet, P., Li, X., Arntz, Y., et al. (2011). Radiopaque iodinated nano-emulsions for preclinical X-ray imaging. *RSC Adv.* 1, 792–801. doi: 10.1039/c1ra00048a

Hardman, R. (2006). A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. *Environ. Health Perspect.* 114, 165–172. doi: 10.1289/ehp.8284

Hare, J. I., Lammers, T., Ashford, M. B., Puri, S., Storm, G., and Barry, S. T. (2017). Challenges and strategies in anti-cancer nanomedicine development: an industry perspective. *Adv. Drug Deliv. Rev.* 108, 25–38. doi: 10.1016/j.addr.2016.04.025

Hartman, K. B., Laus, S., Bolskar, R. D., Muthupillai, R., Helm, L., Toth, E., et al. (2008). Gadonanotubes as ultrasensitive pH-smart probes for magnetic resonance imaging. *Nano Lett.* 8, 415–419. doi: 10.1021/nl0720408

Herth, M. M., Barz, M., Moderegger, D., Allmeroth, M., Jahn, M., Thews, O., et al. (2009). Radioactive labeling of defined HPMA-based polymeric structures using [18F]FETos for *in vivo* imaging by positron emission tomography. *Biomacromolecules* 10, 1697–1703. doi: 10.1021/bm8014736

Hill, M. L., Corbin, I. R., Levitin, R. B., Cao, W., Mainprize, J. G., Yaffe, M. J., et al. (2010). *In vitro* assessment of poly-iodinated triglyceride reconstituted low-density lipoprotein: initial steps toward ct molecular imaging. *Acad. Radiol.* 17, 1359–1365. doi: 10.1016/j.acra.2010.06.006

Ho Kong, W., Jae Lee, W., Yun Cui, Z., Hyun Bae, K., Gwan Park, T., Hoon Kim, J., et al. (2007). Nanoparticulate carrier containing water-insoluble iodinated oil as a multifunctional contrast agent for computed tomography imaging. *Biomaterials* 28, 5555–5561. doi: 10.1016/j.biomaterials.2007.08.044

Holzhausen, C., Gröger, D., Mundhenk, L., Welker, P., Haag, R., and Gruber, A. D. (2013). Tissue and cellular localization of nanoparticles using 35S labeling and light microscopic autoradiography. *Nanomedicine* 9, 465–468. doi: 10.1016/j.nano.2013.02.003

Hong, H., Zhang, Y., Sun, J., and Cai, W. (2009). Molecular imaging and therapy of cancer with radiolabeled nanoparticles. *Nano Today* 4, 399–413. doi: 10.1016/j.nantod.2009.07.001

Hong, Y., Zhu, H., Hu, J., Lin, X., Wang, F., Li, C., et al. (2014). Synthesis and radiolabeling of 111In-core-cross linked polymeric micelle-octreotide for near-infrared fluoroscopy and single photon emission computed tomography imaging. *Bioorg. Med. Chem. Lett.* 24, 2781–2785. doi: 10.1016/j.bmcl.2014.03.050

Hossann, M., Wang, T., Syunyaeva, Z., Wiggenhorn, M., Zengerle, A., Issels, R. D., et al. (2013). Non-ionic Gd-based MRI contrast agents are optimal for encapsulation into phosphatidyldiglycerol-based thermosensitive liposomes. *J. Control. Release* 166, 22–29. doi: 10.1016/j.jconrel.2012.12.003

Hua, S., and Cabot, P. J. (2013). Targeted nanoparticles that mimic immune cells in pain control inducing analgesic and anti-inflammatory actions: a potential novel treatment of acute and chronic pain condition. *Pain Physician* 16, E199–E216.

Jakhmola, A., Anton, N., Anton, H., Messaddeq, N., Hallouard, F., Klymchenko, A., et al. (2014). Poly-ε-caprolactone tungsten oxide nanoparticles as a contrast agent for X-ray computed tomography. *Biomaterials* 35, 2981–2986. doi: 10.1016/j.biomaterials.2013.12.032

Janib, S. M., Moses, A. S., and MacKay, J. A. (2010). Imaging and drug delivery using theranostic nanoparticles. *Adv. Drug Deliv. Rev.* 62, 1052–1063. doi: 10.1016/j.addr.2010.08.004

Jianhua, L., Rui, X., Zhiman, L., Reza, G., Yan, Z., Jishen, Z., et al. (2013). Large-scale and facile synthesis of biocompatible Yb-based nanoparticles as a contrast agent for *in vivo* X-ray computed tomography imaging. *Curr. Top. Med. Chem.* 13, 513–518. doi: 10.2174/1568026611313040011

Jong, W. H., Burger, M. C., Verheijen, M. A., and Geertsma, R. E. (2010). Detection of the presence of gold nanoparticles in organs by transmission electron microscopy. *Materials* 3, 4681–4694. doi: 10.3390/ma3094681

Jung, C. W., and Jacobs, P. (1995). Physical and chemical properties of superparamagnetic iron oxide MR contrast agents: ferumoxides, ferumoxtran, ferumoxsil. *Magn. Reson. Imaging* 13, 661–674. doi: 10.1016/0730-725X(95)00024-B

Kamaly, N., and Miller, A. D. (2010). Paramagnetic liposome nanoparticles for cellular and tumour imaging. *Int. J. Mol. Sci.* 11, 1759–1776. doi: 10.3390/ijms11041759

Kempen, P. J., Thakor, A. S., Zavaleta, C., Gambhir, S. S., and Sinclair, R. (2013). A scanning transmission electron microscopy approach to analyzing large volumes of tissue to detect nanoparticles. *Microsc. Microanal.* 19, 1290–1297. doi: 10.1017/S143192761300192X

Kim, J., Chhour, P., Hsu, J., Litt, H. I., Ferrari, V. A., Popovtzer, R., et al. (2017). Use of nanoparticle contrast agents for cell tracking with computed tomography. *Bioconjug. Chem.* 28, 1581–1597. doi: 10.1021/acs.bioconjchem.7b00194

Kim, J., Kim, H. S., Lee, N., Kim, T., Kim, H., Yu, T., et al. (2008). Multifunctional uniform nanoparticles composed of a magnetite nanocrystal core and a mesoporous silica shell for magnetic resonance and fluorescence imaging and for drug delivery. *Angewand. Chem. Int. Edn.* 47, 8438–8441. doi: 10.1002/anie.200802469

Kobayashi, Y., Imai, J., Nagao, D., Takeda, M., Ohuchi, N., Kasuya, A., et al. (2007). Preparation of multilayered silica–Gd–silica core-shell particles and their magnetic resonance images. *Colloids Surfaces A* 308, 14–19. doi: 10.1016/j.colsurfa.2007.05.024

Koo, V., Hamilton, P. W., and Williamson, K. (2006). Non-invasive *in vivo* imaging in small animal research. *Cell. Oncol.* 28, 127–139. doi: 10.1155/2006/245619

Kumar, R., Ohulchanskyy, T. Y., Turowski, S. G., Thompson, M. E., Seshadri, M., and Prasad, P. N. (2010). Combined magnetic resonance and optical imaging of head and neck tumor xenografts using Gadolinium-labelled phosphorescent polymeric nanomicelles. *Head Neck Oncol.* 2:35. doi: 10.1186/1758-3284-2-35

Kunjachan, S., Ehling, J., Storm, G., Kiessling, F., and Lammers, T. (2015). Noninvasive imaging of nanomedicines and nanotheranostics: principles, progress, and prospects. *Chem. Rev.* 115, 10907–10937. doi: 10.1021/cr500314d

Lammers, T., Kiessling, F., Hennink, W. E., and Storm, G. (2012). Drug targeting to tumors: principles, pitfalls and (pre-) clinical progress. *J. Control. Release* 161, 175–187. doi: 10.1016/j.jconrel.2011.09.063

Leander, P., Höglund, P., Børseth, A., Kloster, Y., and Berg, A. (2001). A new liposomal liver-specific contrast agent for CT: first human phase-I clinical trial assessing efficacy and safety. *Eur. Radiol.* 11, 698–704. doi: 10.1007/s003300000712

Lee, C. C., MacKay, J. A., Fréchet, J. M. J., and Szoka, F. C. (2005). Designing dendrimers for biological applications. *Nat. Biotechnol.* 23:1517–1526. doi: 10.1038/nbt1171

Li, X., Anton, N., Zuber, G., and Vandamme, T. (2014). Contrast agents for preclinical targeted X-ray imaging. *Adv. Drug Deliv. Rev.* 76, 116–133. doi: 10.1016/j.addr.2014.07.013

Liguori, C., Frauenfelder, G., Massaroni, C., Saccomandi, P., Giurazza, F., Pitocco, F., et al. (2015). Emerging clinical applications of computed tomography. *Med. Dev.* 8, 265–278. doi: 10.2147/MDER.S70630

Lin, C., Cai, S., and Feng, J. (2012). Positive contrast imaging of SPIO nanoparticles. *J. Nanomater.* 2012, 1–9. doi: 10.1155/2012/734842

Liu, T. W., MacDonald, T. D., Shi, J., Wilson, B. C., and Zheng, G. (2012). Intrinsically Copper-64-Labeled organic nanoparticles as radiotracers. *Angewand. Chem. Int. Edn.* 51, 13128–13131. doi: 10.1002/anie.201206939

Liu, Y., and Welch, M. J. (2012). Nanoparticles labeled with positron emitting nuclides: advantages, methods, and applications. *Bioconjug. Chem.* 23, 671–682. doi: 10.1021/bc200264c

Liu, Y., Chen, Z., Liu, C., Yu, D., Lu, Z., and Zhang, N. (2011). Gadolinium-loaded polymeric nanoparticles modified with Anti-VEGF as multifunctional MRI contrast agents for the diagnosis of liver cancer. *Biomaterials* 32, 5167–5176. doi: 10.1016/j.biomaterials.2011.03.077

Liu, Y., Tseng, Y. C., and Huang, L. (2012). Biodistribution studies of nanoparticles using fluorescence imaging: a qualitative or quantitative method? *Pharm. Res.* 29, 3273–3277. doi: 10.1007/s11095-012-0818-1

Liu, Z., Cai, W., He, L., Nakayama, N., Chen, K., Sun, X., et al. (2006). *In vivo* biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. *Nat. Nanotechnol.* 2:47–52. doi: 10.1038/nnano.2006.170

Lu, H. D., Wang, L. Z., Wilson, B. K., McManus, S. A., Jumai'an, J., Padakanti, P. K., et al. (2018). Copper loading of preformed nanoparticles for PET-imaging applications. *ACS Appl. Mater. Interfaces* 10, 3191–3199. doi: 10.1021/acsami.7b07242

Lu, P.-L., Chen, Y.-C., Ou, T.-W., Chen, H.-H., Tsai, H.-C., Wen, C.-J., et al. (2011). Multifunctional hollow nanoparticles based on graft-diblock copolymers for doxorubicin delivery. *Biomaterials* 32, 2213–2221. doi: 10.1016/j.biomaterials.2010.11.051

Lusic, H., and Grinstaff, M. W. (2013). X-Ray computed tomography contrast agents. *Chem. Rev.* 113, 1641–1666. doi: 10.1021/cr200358s

Madru, R., Kjellman, P., Olsson, F., Wingårdh, K., Ingvar, C., Ståhlberg, F., et al. (2012). 99mTc-Labeled superparamagnetic iron oxide nanoparticles for multimodality SPECT/MRI of sentinel lymph nodes. *J. Nuclear Med.* 53, 459–463. doi: 10.2967/jnumed.111.092437

Majors, R. (2013). *Sample Preparation Fundamentals for Chromatography*. Mississauga, ON: Agilent Technologies.

Margerum, L. D., Campion, B. K., Koo, M., Shargill, N., Lai, J.-J., Marumoto, A., et al. (1997). Gadolinium(III) DO3A macrocycles and polyethylene glycol coupled to dendrimers effect of molecular weight on physical and biological properties of macromolecular magnetic resonance imaging contrast agents. *J. Alloys Compd.* 249, 185–190. doi: 10.1016/S0925-8388(96)02830-7

Martina, M.-S., Fortin, J.-P., Ménager, C., Clément, O., Barratt, G., Grabielle-Madelmont, C., et al. (2005). Generation of superparamagnetic liposomes revealed as highly efficient MRI contrast agents for *in vivo* imaging. *J. Am. Chem. Soc.* 127, 10676–10685. doi: 10.1021/ja0516460

Massoud, T. F., and Gambhir, S. S. (2003). Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev.* 17, 545–580. doi: 10.1101/gad.1047403

Mayhew, T. M., Mühlfeld, C., Vanhecke, D., and Ochs, M. (2009). A review of recent methods for efficiently quantifying immunogold and other nanoparticles using TEM sections through cells, tissues and organs. *Ann. Anat.* 191, 153–170. doi: 10.1016/j.aanat.2008.11.001

McDevitt, M. R., Chattopadhyay, D., Jaggi, J. S., Finn, R. D., Zanzonico, P. B., Villa, C., et al. (2007). PET Imaging of Soluble Yttrium-86-Labeled carbon nanotubes in mice. *PLoS ONE* 2:e907. doi: 10.1371/journal.pone.0000907

Mercer, R. R., Scabilloni, J., Wang, L., Kisin, E., Murray, A. R., Schwegler-Berry, D., et al. (2008). Alteration of deposition pattern and pulmonary response as a result of improved dispersion of aspirated single-walled carbon nanotubes in a mouse model. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 294, L87–L97. doi: 10.1152/ajplung.00186.2007

Mieszawska, A. J., Mulder, W. J., Fayad, Z. A., and Cormode, D. P. (2013). Multifunctional gold nanoparticles for diagnosis and therapy of disease. *Mol. Pharm.* 10, 831–847. doi: 10.1021/mp3005885

Milane, L., Duan, Z.-F., and Amiji, M. (2011). Pharmacokinetics and biodistribution of lonidamine/paclitaxel loaded, EGFR-targeted nanoparticles in an orthotopic animal model of multi-drug resistant breast cancer. *Nanomedicine* 7, 435–444. doi: 10.1016/j.nano.2010.12.009

Moghimi, S. M., Hunter, A. C., and Andresen, T. L. (2012). Factors controlling nanoparticle pharmacokinetics: an integrated analysis and perspective. *Annu. Rev. Pharmacol. Toxicol.* 52, 481–503. doi: 10.1146/annurev-pharmtox-010611-134623

Morales-Avila, E., Ferro-Flores, G., Ocampo-García, B. E., De León-Rodríguez, L. M., Santos-Cuevas, C. L., García-Becerra, R., et al. (2011). Multimeric system of 99mTc-Labeled gold nanoparticles conjugated to c[RGDfK(C)] for Molecular Imaging of Tumor α(v)β(3) expression. *Bioconjug. Chem.* 22, 913–922. doi: 10.1021/bc100551s

Muddineti, O. S., Ghosh, B., and Biswas, S. (2015). Current trends in using polymer coated gold nanoparticles for cancer therapy. *Int. J. Pharm.* 484, 252–267. doi: 10.1016/j.ijpharm.2015.02.038

Mühlfeld, C., Rothen-Rutishauser, B., Vanhecke, D., Blank, F., Gehr, P., and Ochs, M. (2007). Visualization and quantitative analysis of nanoparticles in the respiratory tract by transmission electron microscopy. *Part. Fibre Toxicol.* 4:11. doi: 10.1186/1743-8977-4-11

Naha, P. C., Zaki, A. A., Hecht, E., Chorny, M., Chhour, P., Blankemeyer, E., et al. (2014). Dextran coated bismuth-iron oxide nanohybrid contrast agents for computed tomography and magnetic resonance imaging. *J. Mater. Chem. B* 2, 8239–8248. doi: 10.1039/C4TB01159G

Oh, M. H., Lee, N., Kim, H., Park, S. P., Piao, Y., Lee, J., et al. (2011). Large-scale synthesis of bioinert tantalum oxide nanoparticles for X-ray computed tomography imaging and bimodal image-guided sentinel lymph node mapping. *J. Am. Chem. Soc.* 133, 5508–5515. doi: 10.1021/ja200120k

Oku, N., Yamashita, M., Katayama, Y., Urakami, T., Hatanaka, K., Shimizu, K., et al. (2011). PET imaging of brain cancer with positron emitter-labeled liposomes. *Int. J. Pharm.* 403, 170–177. doi: 10.1016/j.ijpharm.2010.10.001

Ostrowski, A., Nordmeyer, D., Boreham, A., Holzhausen, C., Mundhenk, L., Graf, C., et al. (2015). Overview about the localization of nanoparticles in tissue and cellular context by different imaging techniques. *Beilstein J. Nanotechnol.* 6, 263–280. doi: 10.3762/bjnano.6.25

Pan, D., Schirra, C. O., Senpan, A., Schmieder, A. H., Stacy, A. J., Roessl, E., et al. (2012). An early investigation of ytterbium nanocolloids for selective and quantitative "Multicolor" spectral CT imaging. *ACS Nano* 6, 3364–3370. doi: 10.1021/nn300392x

Parac-Vogt Tatjana, N., Kimpe, K., Laurent, S., Piérart, C., Elst Luce, V., Muller Robert, N., et al. (2004). Gadolinium DTPA-monoamide complexes incorporated into mixed micelles as possible MRI contrast agents. *Eur. J. Inorg. Chem.* 2004, 3538–3543. doi: 10.1002/ejic.200400187

Park, J. A., Lee, J. J., Jung, J. C., Yu, D. Y., Oh, C., Ha, S., et al. (2008). Gd-DOTA conjugate of RGD as a potential tumor-targeting MRI contrast agent. *Chembiochem* 9, 2811–2813. doi: 10.1002/cbic.200800529

Pavlović, D. M., Babić, S., Horvat, A. J., and Kaštelan-Macan, M. (2007). Sample preparation in analysis of pharmaceuticals. *TrAC Trends Analyt. Chem.* 26, 1062–1075. doi: 10.1016/j.trac.2007.09.010

Peng, X. H., Qian, X., Mao, H., Wang, A. Y., Chen, Z. G., Nie, S., et al. (2008). Targeted magnetic iron oxide nanoparticles for tumor imaging and therapy. *Int. J. Nanomedicine* 3, 311–321.

PerkinElmer (2008). *LSC in Practice: LSC Sample Preparation by Solubilization*. Application Note - PerkinElmer.

Petersen, A. L., Binderup, T., Rasmussen, P., Henriksen, J. R., Elema, D. R., Kjær, A., et al. (2011). 64Cu loaded liposomes as positron emission tomography imaging agents. *Biomaterials* 32, 2334–2341. doi: 10.1016/j.biomaterials.2010.11.059

Peterson, T. E., and Furenlid, L. R. (2011). SPECT detectors: the Anger Camera and beyond. *Phys. Med. Biol.* 56, R145–R182. doi: 10.1088/0031-9155/56/17/R01

Pham, B. T. T., Colvin, E. K., Pham, N. T. H., Kim, B. J., Fuller, E. S., Moon, E. A., et al. (2018). Biodistribution and clearance of stable superparamagnetic maghemite iron oxide nanoparticles in mice following intraperitoneal administration. *Int. J. Mol. Sci.* 19:205. doi: 10.3390/ijms19010205

Pimlott, S. L., and Sutherland, A. (2011). Molecular tracers for the PET and SPECT imaging of disease. *Chem. Soc. Rev.* 40, 149–162. doi: 10.1039/B922628C

Plascencia-Villa, G., Starr, C. R., Armstrong, L. S., Ponce, A., and José-Yacamán, M. (2012). Imaging interactions of metal oxide nanoparticles with macrophage cells by ultra-high resolution scanning electron microscopy techniques. *Integr. Biol.* 4, 1358–1366. doi: 10.1039/c2ib20172k

Plassat, V., Martina, M. S., Barratt, G., Ménager, C., and Lesieur, S. (2007). Sterically stabilized superparamagnetic liposomes for MR imaging and cancer therapy: pharmacokinetics and biodistribution. *Int. J. Pharm.* 344, 118–127. doi: 10.1016/j.ijpharm.2007.05.018

Priem, B., Tian, C., Tang, J., Zhao, Y., and Mulder, W. J. (2015). Fluorescent nanoparticles for the accurate detection of drug delivery. *Expert Opin. Drug Deliv.* 12, 1881–1894. doi: 10.1517/17425247.2015.1074567

Rabin, O., Manuel Perez, J., Grimm, J., Wojtkiewicz, G., and Weissleder, R. (2006). An X-ray computed tomography imaging agent based on long-circulating bismuth sulphide nanoparticles. *Nat. Mater.* 5, 118–122. doi: 10.1038/nmat1571

Reimer, P., and Tombach, B. (1998). Hepatic MRI with SPIO: detection and characterization of focal liver lesions. *Eur. Radiol.* 8, 1198–1204. doi: 10.1007/s003300050535

Richard, C., Doan, B. T., Beloeil, J. C., Bessodes, M., Tóth, É., and Scherman, D. (2008). Noncovalent functionalization of carbon nanotubes with amphiphilic Gd3+ chelates: toward powerful T1 and T2 MRI contrast agents. *Nano Lett.* 8, 232–236. doi: 10.1021/nl072509z

Robson, A. L., Dastoor, P. C., Flynn, J., Palmer, W., Martin, A., Smith, D. W., et al. (2018). Advantages and limitations of current imaging techniques for characterizing liposome morphology. *Front. Pharmacol.* 9:80. doi: 10.3389/fphar.2018.00080

Rongzuo, X., Yanli, W., Xuli, W., Eun-Kee, J., Dennis, L. P., and Zheng-Rong, L. (2007). *In vivo* evaluation of a PAMAM-Cystamine-(Gd-DO3A) conjugate as a biodegradable macromolecular MRI contrast agent. *Exp. Biol. Med.* 232, 1081–1089. doi: 10.3181/0702-RM-33

Rosen, J., Yoffe, S., Meerasa, A., Verma, M., and Gu, F. (2011). Nanotechnology and diagnostic imaging: new advances in contrast agent technology. *J. Nanomedicine Nanotechnol.* 2, 1–12. doi: 10.4172/2157-7439.1000115

Sachse, A., Leike, J. U., Schneider, T., Wagner, S. E., RÖling, G. L., Krause, W., et al. (1997). Biodistribution and computed tomography blood-pool imaging properties of polyethylene glycol-coated iopromide-carrying liposomes. *Invest. Radiol.* 32, 44–50. doi: 10.1097/00004424-199701000-00007

Saito, R., Krauze, M. T., Bringas, J. R., Noble, C., McKnight, T. R., Jackson, P., et al. (2005). Gadolinium-loaded liposomes allow for real-time magnetic resonance imaging of convection-enhanced delivery in the primate brain. *Exp. Neurol.* 196, 381–389. doi: 10.1016/j.expneurol.2005.08.016

Schrand, A. M., Schlager, J. J., Dai, L., and Hussain, S. M. (2010). Preparation of cells for assessing ultrastructural localization of nanoparticles with transmission electron microscopy. *Nat. Protoc.* 5, 744–757. doi: 10.1038/nprot.2010.2

Senpan, A., Caruthers, S. D., Rhee, I., Mauro, N. A., Pan, D., Hu, G., et al. (2009). Conquering the dark side: colloidal iron oxide nanoparticles. *ACS Nano* 3, 3917–3926. doi: 10.1021/nn900819y

Sercombe, L., Veerati, T., Moheimani, F., Wu, S. Y., Sood, A. K., and Hua, S. (2015). Advances and challenges of liposome assisted drug delivery. *Front. Pharmacol.* 6:286. doi: 10.3389/fphar.2015.00286

Shapiro, M. G., Goodwill, P. W., Neogy, A., Yin, M., Foster, F. S., Schaffer, D. V., et al. (2014). Biogenic gas nanostructures as ultrasonic molecular reporters. *Nat. Nanotechnol.* 9, 311–316. doi: 10.1038/nnano.2014.32

Shigematsu, A., Motoji, N., Hatori, A., and Satoh, T. (1995). Progressive application of autoradiography in pharmacokinetic and metabolic studies for the development of new drugs. *Regul. Toxicol. Pharmacol.* 22, 122–142. doi: 10.1006/rtph.1995.1078

Skupin-Mrugalska, P., Sobotta, L., Warowicka, A., Wereszczynska, B., Zalewski, T., Gierlich, P., et al. (2018). Theranostic liposomes as a bimodal carrier for magnetic resonance imaging contrast agent and photosensitizer. *J. Inorg. Biochem.* 180, 1–14. doi: 10.1016/j.jinorgbio.2017.11.025

Smith, C. E., Shkumatov, A., Withers, S. G., Yang, B., Glockner, J. F., and Misra, S., et al. (2013). A polymeric fastener can easily functionalize liposome surfaces with gadolinium for enhanced magnetic resonance imaging. *ACS Nano* 7, 9599–9610, 10.1021/nn4026228

Strijkers, G. J., Mulder, W. J., van Tilborg, G. A., and Nicolay, K. (2007). MRI contrast agents: current status and future perspectives. *Anticancer Agents Med. Chem.* 7, 291–305. doi: 10.2174/187152007780618135

Sun, C., Lee, J. S., and Zhang, M. (2008). Magnetic nanoparticles in MR imaging and drug delivery. *Adv. Drug Deliv. Rev.* 60, 1252–1265. doi: 10.1016/j.addr.2008.03.018

Sun, X., Cai, W., and Chen, X. (2015). Positron emission tomography imaging using radiolabeled inorganic nanomaterials. *Acc. Chem. Res.* 48, 286–294. doi: 10.1021/ar500362y

Tasciotti, E., Godin, B., Martinez, J. O., Chiappini, C., Bhavane, R., Liu, X., et al. (2011). Near-infrared imaging method for the *in vivo* assessment of the biodistribution of nanoporous silicon particles. *Mol. Imaging* 10, 56–68. doi: 10.2310/7290.2011.00011S1

Taylor, K. M., Kim, J. S., Rieter, W. J., An, H., Lin, W., and Lin, W. (2008). Mesoporous silica nanospheres as highly efficient MRI contrast agents. *J. Am. Chem. Soc.* 130, 2154–2155. doi: 10.1021/ja710193c

Tilcock, C., Unger, E., Cullis, P., and MacDougall, P. (1989). Liposomal Gd-DTPA: preparation and characterization of relaxivity. *Radiology* 171, 77–80. doi: 10.1148/radiology.171.1.2928549

Titford, M. (2009). Progress in the development of microscopical techniques for diagnostic pathology. *J. Histotechnol.* 32, 9–19. doi: 10.1179/his.2009.32.1.9

Torchilin, V. P. (2002). PEG-based micelles as carriers of contrast agents for different imaging modalities. *Adv. Drug Deliv. Rev.* 54, 235–252. doi: 10.1016/S0169-409X(02)00019-4

Torchilin, V. P., Frank-Kamenetsky, M. D., and Wolf, G. L. (1999). CT visualization of blood pool in rats by using long-circulating, iodine-containing micelles. *Acad. Radiol.* 6, 61–65. doi: 10.1016/S1076-6332(99)80063-4

Townsend, D. W. (2004). Physical principles and technology of clinical PET imaging. *Ann. Acad. Med. Singap.* 33, 133–145.

Tsuchiya, K., Nitta, N., Sonoda, A., Nitta-Seko, A., Ohta, S., Otani, H., et al. (2011). Histological study of the biodynamics of iron oxide nanoparticles with different diameters. *Int. J. Nanomedicine* 6, 1587–1594. doi: 10.2147/IJN.S22189

Tu, C., Ma, X., House, A., Kauzlarich, S. M., and Louie, A. Y. (2011). PET imaging and biodistribution of silicon quantum dots in mice. *ACS Med. Chem. Lett.* 2, 285–288. doi: 10.1021/ml1002844

Unger, E. C., Winokur, T., MacDougall, P., Rosenblum, J., Clair, M., Gatenby, R., et al. (1989). Hepatic metastases: liposomal Gd-DTPA-enhanced MR imaging. *Radiology* 171, 81–85. doi: 10.1148/radiology.171.1.2928550

Van Audenhaege, K., Van Holen, R., Vandenberghe, S., Vanhove, C., Metzler, S. D., and Moore, S. C. (2015). Review of SPECT collimator selection, optimization, and fabrication for clinical and preclinical imaging. *Med. Phys.* 42, 4796–4813. doi: 10.1118/1.4927061

Vats, M., Mishra, S. K., Baghini, M. S., Chauhan, D. S., Srivastava, R., and De, A. (2017). Near Infrared Fluorescence Imaging in Nano-Therapeutics and Photo-Thermal Evaluation. *Int. J. Mol. Sci.* 18:924. doi: 10.3390/ijms18050924

Veiseh, O., Sun, C., Fang, C., Bhattarai, N., Gunn, J., Kievit, F., et al. (2009). Specific targeting of brain tumors with an optical/MR imaging nanoprobe across the blood brain barrier. *Cancer Res.* 69, 6200–6207. doi: 10.1158/0008-5472.CAN-09-1157

Wang, H., Zheng, L., Peng, C., Guo, R., Shen, M., Shi, X., et al. (2011). Computed tomography imaging of cancer cells using acetylated dendrimer-entrapped gold nanoparticles. *Biomaterials* 32, 2979–2988. doi: 10.1016/j.biomaterials.2011.01.001

Wiegers, C. B., Welch, M. J., Sharp, T. L., Brown, J. J., Perman, W. H., Sun, Y., et al. (1992). Evaluation of two new gadolinium chelates as contrast agents for MRI. *Magn. Reson. Imaging* 10, 903–911. doi: 10.1016/0730-725X(92)90444-5

Wu, W., Li, R., Bian, X., Zhu, Z., Ding, D., Li, X., et al. (2009). Covalently combining carbon nanotubes with anticancer agent: preparation and antitumor activity. *ACS Nano* 3, 2740–2750. doi: 10.1021/nn9005686

Xiao, T., Wen, S., Wang, H., Liu, H., Shen, M., Zhao, J., et al. (2013). Facile synthesis of acetylated dendrimer-entrapped gold nanoparticles with enhanced gold loading for CT imaging applications. *J. Mater. Chem. B* 1, 2773–2780. doi: 10.1039/c3tb20399a

Xiao, Y., Hong, H., Javadi, A., Engle, J. W., Xu, W., Yang, Y., et al. (2012). Multifunctional unimolecular micelles for cancer-targeted drug delivery and positron emission tomography imaging. *Biomaterials* 33, 3071–3082. doi: 10.1016/j.biomaterials.2011.12.030

Xie, H., Wang, Z. J., Bao, A., Goins, B., and Phillips, W. T. (2010). *In vivo* PET imaging and biodistribution of radiolabeled gold nanoshells in rats with tumor xenografts. *Int. J. Pharm.* 395, 324–330. doi: 10.1016/j.ijpharm.2010.06.005

Yang, X., Hong, H., Grailer, J. J., Rowland, I. J., Javadi, A., Hurley, S. A., et al. (2011). cRGD-functionalized, DOX-conjugated, and (64)Cu-Labeled superparamagnetic iron oxide nanoparticles for targeted anticancer drug delivery and PET/MR imaging. *Biomaterials* 32, 4151–4160. doi: 10.1016/j.biomaterials.2011.02.006

Yordanov, A. T., Lodder, A. L., Woller, E. K., Cloninger, M. J., Patronas, N., Milenic, D., et al. (2002). Novel iodinated dendritic nanoparticles for Computed Tomography (CT) imaging. *Nano Lett.* 2, 595–599. doi: 10.1021/nl020246x

You, J., Zhang, R., Xiong, C., Zhong, M., Melancon, M., Gupta, S., et al. (2012). Effective photothermal chemotherapy using doxorubicin-loaded gold nanospheres that target EphB4 receptors in tumors. *Cancer Res.* 72, 4777–4786. doi: 10.1158/0008-5472.CAN-12-1003

Yuan, H., Wilks, M. Q., Normandin, M. D., El Fakhri, G., Kaittanis, C., and Josephson, L. (2018). Heat-induced radiolabeling and fluorescence labeling of Feraheme nanoparticles for PET/SPECT imaging and flow cytometry. *Nat. Protoc.* 13, 392–412. doi: 10.1038/nprot.2017.133.

Zhang, X., Bloch, S., Akers, W., and Achilefu, S. (2012). Near-infrared molecular probes for *in vivo* imaging. *Curr. Protoc. Cytom.* 60, Chapter 12:Unit 12.27. doi: 10.1002/0471142956.cy1227s6

Zhang, Y., Sun, Y., Xu, X., Zhang, X., Zhu, H., Huang, L., et al. (2010a). Synthesis, biodistribution, and microsingle photon emission computed tomography (SPECT) imaging study of Technetium-99m labeled PEGylated Dendrimer Poly(amidoamine) (PAMAM)–folic acid conjugates. *J. Med. Chem.* 53, 3262–3272. doi: 10.1021/jm901910j

Zhang, Y., Sun, Y., Xu, X., Zhu, H., Huang, L., Zhang, X., et al. (2010b). Radiosynthesis and micro-SPECT imaging of 99mTc-dendrimer poly(amido)-amine folic acid conjugate. *Bioorg. Med. Chem. Lett.* 20, 927–931. doi: 10.1016/j.bmcl.2009.12.075

Zhao, M. X., and Zeng, E. Z. (2015). Application of functional quantum dot nanoparticles as fluorescence probes in cell labeling and tumor diagnostic imaging. *Nanoscale Res. Lett.* 10:171. doi: 10.1186/s11671-015-0873-8

Zhao, Y., Sultan, D., Detering, L., Cho, S., Sun, G., Pierce, R., et al. (2014). Copper-64-alloyed gold nanoparticles for cancer imaging: improved radiolabel stability and diagnostic accuracy. *Angewand. Chem. Int. Edn.* 53, 156–159. doi: 10.1002/anie.201308494

Zheng, J., Petty, J. T., and Dickson, R. M. (2003). High Quantum Yield Blue Emission from Water-Soluble Au8 Nanodots. *J. Am. Chem. Soc.* 125, 7780–7781. doi: 10.1021/ja035473v

Zhou, J., Lu, Z., Shan, G., Wang, S., and Liao, Y. (2014). Gadolinium complex and phosphorescent probe-modified NaDyF4 nanorods for T1- and T2-weighted MRI/CT/phosphorescence multimodality imaging. *Biomaterials* 35, 368–377. doi: 10.1016/j.biomaterials.2013.09.088

Zhou, Z., and Lu, Z.-R. (2013). Gadolinium-based contrast agents for MR cancer imaging. *Wiley Interd. Rev. Nanomedicine Nanobiotechnol.* 5, 1–18. doi: 10.1002/wnan.1198

Ziegler, S. I. (2005). Positron emission tomography: principles, technology, and recent developments. *Nuclear Physics A* 752:679–687. doi: 10.1016/j.nuclphysa.2005.02.067

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Advantages and Limitations of Current Imaging Techniques for Characterizing Liposome Morphology

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There are currently a number of imaging techniques available for evaluating the morphology of liposomes and other nanoparticles, with each having its own advantages and disadvantages that should be considered when interpreting data. Controlling and validating the morphology of nanoparticles is of key importance for the effective clinical translation of liposomal formulations. There are a number of physical characteristics of liposomes that determine their *in vivo* behavior, including size, surface characteristics, lamellarity, and homogeneity. Despite the great importance of the morphology of nanoparticles, it is generally not well-characterized and is difficult to control. Appropriate imaging techniques provide important details regarding the morphological characteristics of nanoparticles, and should be used in conjunction with other methods to assess physicochemical parameters. In this review, we will discuss the advantages and limitations of available imaging techniques used to evaluate liposomal formulations.

Keywords: liposomes, microscopy, imaging, nanoparticles, light microscopy, electron microscopy, atomic-force microscopy

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INTRODUCTION

Liposomes are a type of nanocarrier that have been widely investigated for drug-delivery purposes. They are composed of phospholipid bilayers which enclose a distinct aqueous space, thereby allowing encapsulation of both hydrophilic and hydrophobic compounds (Metselaar and Storm, 2005). Liposomes are able to stabilize therapeutic compounds and overcome barriers to cellular and tissue uptake (Ding et al., 2006; Hua and Wu, 2013). This allows them to improve targeting of compounds to sites of disease and consequently reduce accumulation in non-target organs (Bakker-Woudenberg et al., 1994; Mastrobattista et al., 1999; Hua, 2013; Hua et al., 2015; Sercombe et al., 2015; Zununi Vahed et al., 2017). There are four main types of liposomes based on their surface characteristics – conventional liposomes, PEGylated liposomes, ligand-targeted liposomes, and theranostic liposomes (**Figure 1**; Sercombe et al., 2015). Ligand-targeted liposomes provide the potential for site-specific delivery of drugs to certain tissues or organs that selectively express the targeted ligand (Willis and Forssen, 1998; Bendas, 2001; Sawant and Torchilin, 2012), whereas PEGylated liposomes confer steric hindrance to enhance the circulation half-life of the delivery system following systemic administration (Torchilin, 1994; Wang et al., 2015). Liposomes

incorporating a combination of the various delivery platforms can further improve the delivery of encapsulated compounds, depending on the route of administration and site of disease.

Following the manufacturing process, liposomes are characterized to ensure homogeneity across a number of parameters, including drug encapsulation, ligand-conjugation, lipid composition, surface charge, and morphological properties (e.g., size, shape, and number of lamellae) (Kuntsche et al., 2011). These characteristics are important as they can have a major impact on the behavior of liposomes both *in vitro* and *in vivo* (Sawant and Torchilin, 2012; Sercombe et al., 2015). Recognition and clearance of liposomes by the body's defenses, including the reticuloendothelial system (RES) and adsorption of opsonins with subsequent uptake by the mononuclear phagocytic system, are major contributors to the clearance and degradation of liposomes (Senior, 1987; Cullis et al., 1998; Ishida et al., 2001). Therefore, being able to determine the physicochemical properties of manufactured liposomes is important to optimize a formulation for further translational evaluation.

A major aspect in the physicochemical assessment of liposomes is visualizing the morphology of the nanoparticles using microscopy. There are a number of techniques available for imaging liposomes and other nanoparticles that can be broadly categorized into light, electron, or atomic-force microscopy (Bibi et al., 2011). Each technique has its own advantages and limitations, which should be considered when evaluating studies on nanoparticle-based drug delivery systems **(Table 1)**. This review will evaluate each imaging technique used to assess the morphological characteristics of liposomes.

LIGHT MICROSCOPY

Light or optical microscopy refers to microscopes that utilize visible light and an arrangement of lenses to magnify a field of view (Murphy and Davidson, 2012c). Basic light microscopy itself is unable to provide comprehensive information about the lipid bilayer compared to the detail offered by other microscopy techniques. However, it can be used to rapidly obtain an image of vesicles using basic laboratory equipment (Bibi et al., 2011). This technique can be particularly useful when gathering general information on the size, shape, homogeneity, and degree of aggregation of a liposome sample (Nallamothu et al., 2006). Light microscopes have an ultimate resolution of ∼250 nm (governed by the smallest diffraction-limited spot size that can be achieved by the instrument) and, as such, are typically incapable of providing detailed information regarding the structures of small unilamellar vesicles (SUVs) and the lamellarity of vesicles (Bibi et al., 2011). Generally, light microscopy can only provide significant information on giant unilamellar vesicles (GUVs), which can range from single to hundreds of micrometers in diameter (Bagatolli, 2009). Incorporation of fluorescent probes, polarization techniques, and application of high-resolution confocal microscopy can provide more information about the 3D structure and lamellarity of the vesicles (Bagatolli, 2009).

Polarization Microscopy

This type of light microscopy utilizes the unique ability of polarized light to interact with polarizable bonds of ordered molecules (Murphy and Davidson, 2012d). Enhanced light absorption occurs when molecular dipoles in the sample are aligned with the polarization vector of the incident light wave; resulting in phase differences between sampling light rays, which in turn produce interference-dependent changes in amplitude in the image plane (Murphy and Davidson, 2012d). Image contrast then arises not only from the effects of interference and diffraction, but also due to the presence of ordered molecular arrangements (Murphy and Davidson, 2012d). It can be used to study the form and dynamics of many ordered cellular structures, including lipid bilayers of plasma membranes (Bibi et al., 2011; Murphy and Davidson, 2012d). Images can be obtained in either monochrome or color. This technique provides an alternative method to visualize liposomes, particularly to confirm the formation of vesicles. It does not provide conclusive observations regarding the bilayer characteristics or lamellarity of the vesicles (Bibi et al., 2011). In addition, polarization microscopy is also limited by the size of the vesicles that can be visualized, with large vesicles in the micrometer range having the optimal clarity. This technique does not provide clear images of particles in the nanometer range.

Fluorescence Microscopy

Fluorescence or "epifluorescence" microscopy is a special form of light microscopy that exploits the ability of fluorochromes to emit light after being excited with light of a certain wavelength (Murphy and Davidson, 2012b). This technique is widely used in biophysics to provide essential information about the structure and dynamics of membrane components (Bouvrais et al., 2010). In these studies, fluorescent probes are specifically incorporated into the membrane under investigation, permitting visualization of the structure. A large range of fluorescent dyes with various chemical and photonic properties are commercially available, catering to a variety of research questions. For example, certain fluorescent dyes may prefer specific membrane environments exhibiting different arrangements and lateral packing (Bouvrais et al., 2010). As such, fluorescence microscopy allows for the potential to simultaneously apply multiple probes within a sample to provide information about the membrane structure itself. The fluorescent probes can be placed within the aqueous compartment as well as the lipid bilayer of liposomes (Bibi et al., 2011). This arrangement can be especially useful when viewing GUVs, where information can be obtained regarding the shape, size and fluidity of the lipid vesicles (Klymchenko et al., 2009; Bouvrais et al., 2010). Incorporation of probes, such as rhodamine-labeled lipids, directly into the lipid bilayer can also allow visualization of the lamellarity of liposomes (Bibi et al., 2011).

The disadvantage of fluorescent microscopy is that the addition of probes and dyes to a membrane system can potentially interfere with the properties of the liposomal delivery system (Bouvrais et al., 2010; Bibi et al., 2011; Murphy and Davidson, 2012b). However, the use of low dye concentrations

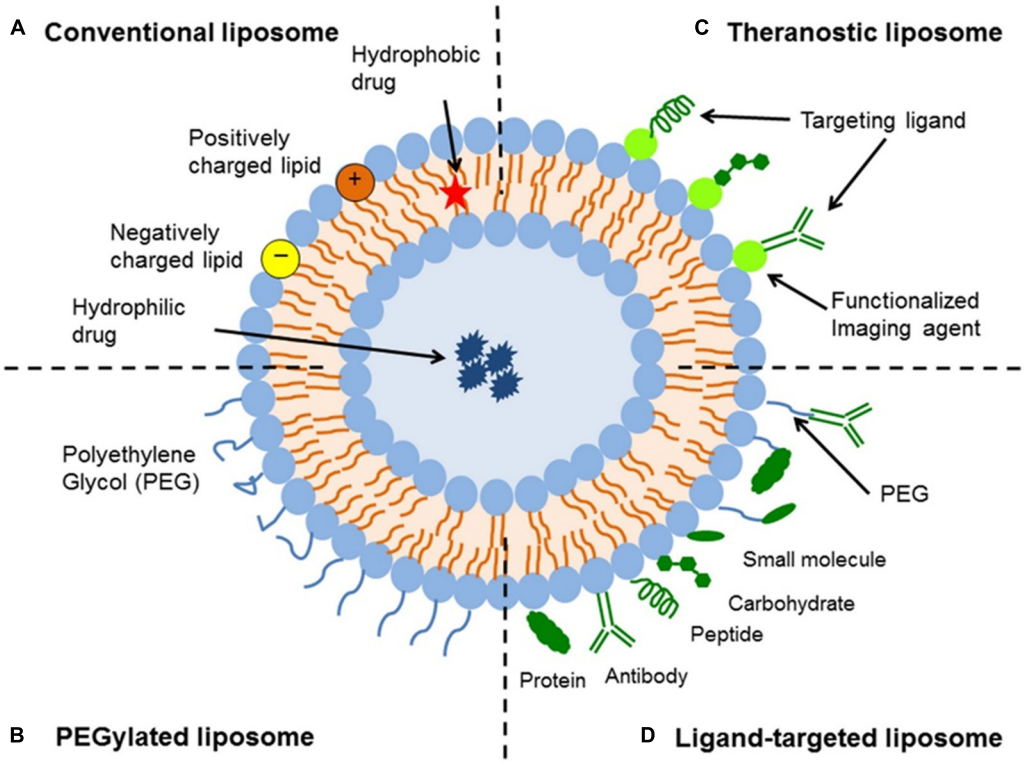

FIGURE 1 | Schematic representation of the different types of liposomal drug delivery systems – **(A)** Conventional liposome, **(B)** PEGylated liposome, **(C)** Ligand-targeted liposome, and **(D)** Theranostic liposome (Sercombe et al., 2015).

(≤1 mol%) has been shown to have minimal impact on the physical properties of the membrane (Bouvrais et al., 2010). It is also important to note that the choice of fluorescent dye is a critical step, as some dyes can induce large changes in the host membrane and/or cause experimental artifacts, resulting in inaccurate data interpretation (Bouvrais et al., 2010). In addition, photo-induced lipid peroxidation can also lead to domain formation even in simple dye systems. This process can result in the formation of large rafts and spontaneous facet formation (Bouvrais et al., 2010). Prolonged exposure to fluorescent light can also result in bleaching and loss of fluorescence intensity. Therefore, the choice of fluorescent dye and the development of new membrane probes are important considerations when using fluorescence microscopy (Klymchenko et al., 2009).

Confocal Microscopy

Confocal scanning microscopy has been an advancement in the area of fluorescence microscopy. Rather than illuminating the entire sample, an image is built by scanning one (or more) focused beams of light across the sample. Light returning from the illuminated sample passes through an aperture that rejects out-of-focus light from above and below the plane of interest; ensuring that only images from a small depth of field are obtained, greatly improving the out-of-plane resolution (Bibi et al., 2011; Murphy and Davidson, 2012a). Using this technique, a "z-stack" of images is collected, starting from the top of the vesicle followed by images taken in defined z-increments to the bottom of the sample, resulting in a composite 3D image of the sample (Bibi et al., 2011; Murphy and Davidson, 2012a). Confocal scanning microscopy has become a more attractive technique over epifluorescent light microscopes due to its superior image clarity. In the case of GUVs, this technique is capable of visualizing the internal structure of the lipid systems, which is often not possible with other microscopy methods (Ruozi et al., 2011; Mertins and Dimova, 2013). For example, separation of the aqueous and lipid bilayer phase can be clearly visualized in larger vesicles (Mertins and Dimova, 2013). However, confocal microscopy is still diffraction-limited and, therefore, unable to produce high-definition images of SUVs or oligolamellar liposomes (Ruozi et al., 2011).

ELECTRON MICROSCOPY TECHNIQUES

Electron microscopy is a method for the visualization of vesicles under very high magnification (Henry, 2005). It is widely used in the characterization of lipid vesicles as the electron wavelength (and hence diffraction-limited resolution), is many orders of magnitude lower than that of optical microscopy, and therefore provides super-resolution for clear visualization of small liposomes (Bibi et al., 2011; Ruozi et al., 2011). This technique uses a beam of electrons focused onto the surface

TABLE 1 | Summary of current imaging techniques for characterizing liposome morphology.

Technique	Advantages	Disadvantages
Basic light microscopy	• Rapid and simple • Provides general information on the size, shape, homogeneity, and degree of aggregation, particularly for GUVs	• Unable to provide comprehensive information about the lipid bilayer, especially for SUVs
Polarization microscopy	• Provides an alternative method to confirm the formation of vesicles • Optimal clarity for large vesicles in the micrometer range	• Unable to provide conclusive observations regarding the bilayer characteristics or lamellarity of the vesicles
Fluorescence microscopy	• Especially useful when viewing GUVs, where information can be obtained regarding the shape, size, and fluidity of the lipid vesicles • Can apply multiple probes within a sample to provide information about the membrane structure itself	• Addition of probes and dyes can potentially interfere with the properties of the lipid vesicles and/or cause experimental artifacts, resulting in inaccurate data interpretation • Photo-induced lipid peroxidation can lead to domain formation • Prolonged exposure to fluorescent light can result in bleaching and loss of fluorescence intensity
Confocal microscopy	• Superior image clarity over fluorescence microscopy • Can provide a composite 3D image of the sample • Capable of visualizing the internal structure of lipid vesicles, particularly for GUVs	• Unable to produce high definition images of SUVs or oligolamellar liposomes
Scanning electron microscopy	• Allows visualization of small vesicles under very high magnification • Provides general detail on the size and spherical morphology of lipid vesicles	• Unable to provide detailed information on the lamellarity and internal structure of lipid vesicles • Liposome structure may suffer perturbations due to the high-vacuum conditions and staining processes required prior to imaging
Transmission electron microscopy *Negative staining technique*	• Provides much higher magnification for imaging nanoparticles, including SUVs • Provides information on morphology, size distribution, homogeneity, and surface structure	• Vesicles are in direct contact with the grid, which may affect their orientation and morphology • Placing the sample under vacuum can cause further dehydration of the sample • Sample preparation can cause changes to the original liposome structure and lead to the creation of light and dark fringes that may be mistaken for lamellar structures
Transmission electron microscopy *Freeze–fracture technique*	• Provides much higher magnification for imaging nanoparticles, including SUVs • Does not require any drying process • Provides detailed information on the 3D structure of the vesicles and bilayer organization • Replicas closely reflect the original native state of the sample	• Artifacts may still occur in the sample during preparation due to insufficient freezing rate, re-deposition of solvent molecules and/or mechanical stress
Transmission electron microscopy *Cryogenic TEM*	• Most useful form of microscopy currently available to study liposomes • Allows for the analysis of liposomes in their most native state • Avoids issues with chemical fixation, dehydration, cutting, and staining • Provides detailed information on the size, shape, internal structure, and lamellarity of liposomes • Sample preparation minimizes the formation of ice crystals and preserves proteins or other materials • Resolution range is ∼5 to 500 nm, as defined by the thickness of the film	• Utilizes lower doses of electrons, which often results in lower resolution compared to other TEM methods • Artifacts are still possible due to the formation of a thin film of amorphous ice and the use of blotting on the sample applying shear forces during the film formation
Environmental scanning electron microscopy	• Allows visualization of small vesicles under very high magnification • Provides general information on the size and shape of lipid vesicles • Allows imaging of dynamic changes of wet systems without previous sample preparation • Does not require the use of fixing, staining or freezing of vesicles • Able to modify sample environment, including pressure, temperature and gas compositions	• Unable to provide detailed information on the internal structure of lipid vesicles

(Continued)

TABLE 1 | Continued

Technique	Advantages	Disadvantages
Atomic force microscopy	• Outstanding resolution in the order of fractions of a nanometer • Provides 3D imaging of liposomes and details on morphology, size distribution, homogeneity, stability, and surface structure • Does not need to operate in a vacuum and can operate in ambient air or under liquid • Can provide information about the mechanical and chemical properties of a sample surface through force measurements	• Requires nanoparticles to be adsorbed onto support surfaces, which can modify the size and shape of the vesicles • Periodic contact of the probing tip can drag the liposomes as it moves across the vesicles in a sample

of the sample by various electromagnetic lenses. The electrons are then scattered by the sample, and are then refocused and magnified by a further series of electromagnetic lenses in the imaging column to produce a projected image (Henry, 2005). There are a number of different types of electron microscopes, each requiring a different sample preparation method.

Scanning Electron Microscopy (SEM)

Scanning electron microscopes (SEMs) utilize an electron beam that is scanned across or over a sample (rather than through a sample) to produce a magnified image of an object (Adler and Schiemann, 1985). Alder et al. first attempted to use SEM to characterize liposomes in 1984 (Adler and Schiemann, 1985). They showed that using the freeze-drying method to prepare the liposome samples for SEM resulted in a large proportion of visible lumps and crusted material (Adler and Schiemann, 1985). More recent studies have shown that the liposome structure itself may suffer perturbations due to the high-vacuum conditions and staining processes required for this preparation technique (Ruozi et al., 2011). SEM is now not commonly used for analyzing liposomes because it requires the sample to be dried or fixed prior to imaging (Ruozi et al., 2011). However, SEM can provide general information on the concentric structure of the different lipid layers, as well as give detail on the size and spherical morphology of a preparation (Nirale et al., 2009).

Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is the most frequently used imaging method for the evaluation of the structure of nanoparticles (Henry, 2005; Kuntsche et al., 2011). It involves the preparation of a thin sample (<100 nm thick) that is placed in a vacuum chamber. The electron beam crosses the sample, where it is then focused by the objective lens to form an image. TEM can readily image soft-matter samples with a spatial resolution down to well below 1 nm in size (Henry, 2005; Kuntsche et al., 2011; Ruozi et al., 2011). This technique can be further categorized based on the sample-preparation method utilized – in particular, negative staining, freeze–fracture and cryogenic TEM. It can provide information on surface modifications of nanoparticles as it provides better contrast and contour of images than other microscopy techniques. For example, conjugation of transferrin to the surface of DSPC/cholesterol liposomes showed a particulate surface coating with negative-stain TEM, which was absent in the unconjugated liposome preparations (Anabousi et al., 2005). In a separate experiment, specific antibodies raised against human transferrin were added before the negative stain procedure to confirm the identity of the particulate surface coating (Anabousi et al., 2005). Therefore, TEM provides the possibility to achieve much-improved resolution when assessing the conjugation of ligands to the surface of liposomes.

Negative Staining Technique

In negative-stain TEM, sample preparation involves a small amount of hydrated sample being placed onto a grid. As the vesicles are in direct contact with the grid, their orientation and morphology may be affected, and hence this should be taken into account (Bibi et al., 2011). It is also necessary to embed the liposomes in a suitable electron-dense material (e.g., heavy metal salts like uranyl acetate or phosphotungstic acid) that provides high contrast, so vesicles can be viewed against a dark-stained background (Ruozi et al., 2011). The negative-staining technique is relatively fast and simple; however, it has been shown to cause changes to the original liposome structure and can lead to the creation of light and dark fringes that may be mistaken for lamellar structures (Bibi et al., 2011; Ruozi et al., 2011). In addition, placing the sample under vacuum can cause further dehydration of the sample, which can again cause changes in the structure of the vesicles (Bibi et al., 2011). Whilst the negative-stain TEM technique can provide much higher magnification for imaging nanoparticles, the damage to the liposome structure makes it difficult to accurately evaluate the morphological characteristics of the sample. Therefore, other imaging techniques are generally required to confirm results.

Freeze–Fracture Technique

The freeze–fracture technique does not require any drying process and can provide additional information about the internal structure of nanoparticles (Kuntsche et al., 2011). This method involves placing a sample on a TEM grid that is sandwiched between two copper or gold holders (Severs, 2007). The sample is vitrified via rapid freezing, typically with liquid propane or liquid nitrogen, before being fractured along areas of the sample with weak molecular interactions. This fracture surface can be further etched and shadowed with a thin platinum or carbon layer to provide a "negative" replica of the fracture sample plane (Severs, 2007; Kuntsche et al., 2011). The replica is then cleaned with an organic solvent to remove all residues

prior to visualization under a TEM microscope. As these replicas are so stable, they can be stored and viewed later (Kuntsche et al., 2011). The major advantage of this technique is that the replicas closely reflect the native state of the sample, and can provide detailed information on the 3D structure of the vesicles and bilayer organization (Bibi et al., 2011; Kuntsche et al., 2011). This information includes the multilamellar construction and bilayer packing of multilamellar vesicles. This technique can also assess aggregate size and may be particularly useful for examining the interaction of cationic liposomes with DNA (Bibi et al., 2011). However, artifacts may still occur in the sample during preparation due to insufficient freezing rate, re-deposition of solvent molecules and/or mechanical stress (Severs, 2007; Kuntsche et al., 2011). For example, a "rippling effect" can occur on the SUVs in the sample (Bibi et al., 2011). This is a common bilayer deformation that is due to a disorder in the transitions of the acyl chains prior to freezing. Incubating vesicles between the pre-transition and actual transition temperature can also cause ripples (Bibi et al., 2011). Interestingly, this can be used to provide information into the lipid phase transitions that occur with the varying nature of lipids used for liposomal preparations (Bibi et al., 2011).

Cryogenic TEM

Cryogenic TEM (cryo-TEM) is a variation of TEM where thin aqueous hydrated films which are vitrified in liquid ethane are used prior to imaging. This technique allows for the analysis of liposomes in their most native state, and is a valuable tool to determine the size, shape, internal structure, and lamellarity of liposomes (Weisman et al., 2004; Bibi et al., 2011; Kuntsche et al., 2011). The major advantage of rapidly freezing liposome samples is minimizing the formation of ice crystals and preserving proteins or other materials (Bibi et al., 2011; Kuntsche et al., 2011). This is beneficial where proteins or DNA have been encapsulated within the nanoparticles (Weisman et al., 2004; Kuntsche et al., 2011). Cryo-TEM is the most useful form of microscopy currently available to study liposomes, as it avoids issues with chemical fixation, dehydration, cutting and staining – all of which can affect the morphology of vesicles (Bibi et al., 2011). The resolution range is ∼5 to 500 nm, as defined by the thickness of the film (Almgren et al., 2000). However, several limitations should be noted with this technique, including the fact that only a 2D image is obtained from 3D objects (which generally also applies to most of the other microscopy techniques). To overcome this limitation, reconstruction of 3D shapes from a sufficiently large number of 2D images of randomly oriented non-spherical particles is possible (Orlova et al., 1999), as well as viewing the sample at different tilt angles to attain information about 3D shape (Van Antwerpen and Gilkey, 1994). Cryogenic electron tomography (cryo-ET) can also be used instead to attain 3D images (Le Bihan et al., 2009). Cryo-TEM also utilizes lower doses of electrons, which means that it often has a lower resolution compared to other methods (Bibi et al., 2011; Kuntsche et al., 2011). Artifacts are still possible due to the formation of a thin film of amorphous ice and the use of blotting on the sample applying shear forces during the film formation (Almgren et al., 2000; Bibi et al., 2011).

Environmental Scanning Electron Microscopy (ESEM)

Environmental scanning electron microscopy (ESEM) is an imaging system that does not require the use of fixing, staining or freezing of vesicles, and can allow imaging of dynamic changes of wet systems without previous sample preparation (Muscariello et al., 2005; Ruozi et al., 2011). The main feature of ESEM is the presence of water vapor in the microscope chamber. The ability to maintain a water-containing atmosphere around the sample that may be partially or even fully hydrated is made possible by the use of a multiple-aperture, graduated vacuum system that allows the imaging chamber to be sustained at pressures up to 55 hPa (Bibi et al., 2011; Ruozi et al., 2011). The primary electron beam can generate secondary electrons that then encounter vapor molecules, leading to a cascade amplification of the signal before reaching the detector. Because of this, ESEM does not require sample preparation (Muscariello et al., 2005). This technique allows for variation in the sample environment through a series of pressure, temperature and gas compositions (Mohammed et al., 2004), which is useful when determining how environmental changes affect the vesicles. This is applicable to nanopharmaceutical formulation and stability studies (Bibi et al., 2011). ESEM has also been used to analyze drug loading into the bilayer of liposomes (Mohammed et al., 2004), as well as determining the size and shape of vesicles. A limitation of ESEM is that it cannot provide detailed information regarding the lamellarity and internal architecture of the nanoscale structures (Ruozi et al., 2011).

ATOMIC FORCE MICROSCOPY (AFM)

Atomic force microscopy (AFM), also known as scanning-force microscopy (SFM), is a type of scanning probe microscope technique. It works by running a sharp tip attached to a cantilever and sensor over the surface of a sample and measuring the surface forces between the probe and the sample (Sitterberg et al., 2010). As the cantilever runs along the sample surface, it moves up and down due to the surface features and the cantilever deflects accordingly. This deflection is usually quantified using an optical sensor, with the laser beam being reflected on the back of the cantilever onto the light detector (Sitterberg et al., 2010). AFM does not need to operate in a vacuum and can operate in ambient air or under liquid; hence it is increasingly being used to image biological samples as well as nanoparticles (Liang et al., 2004a,b; Ruozi et al., 2005, 2009). AFM has outstanding resolution in the order of fractions of a nanometer and can provide a 3D image of liposomes along with details on morphology, size distribution, homogeneity, and stability (Liang et al., 2004a,b; Ruozi et al., 2005, 2007, 2009). Importantly, AFM can be used to characterize the surface modifications of liposomes and detect ligands (e.g., antibodies and polymers) conjugated at the liposomal membrane surface (Bendas et al., 1999; Moutardier et al., 2003;

Anabousi et al., 2005; Liang et al., 2005). For example, Bendas et al. used AFM to magnify the liposomal membrane border and were able to image trimeric structures, approximately 8–10 nm in diameter, which represented the coupling of IgG antibodies to the liposome surface (Bendas et al., 1999). The findings showed that the effectiveness of the technique was highly dependent on the conjugation method used, with antibodies conjugated directly to the liposomal surface being visible with AFM (restricted protein mobility), in comparison to antibodies attached to PEG chains. It was suggestive that the PEG chains caused high protein mobility and, therefore, were unable to be scanned (Bendas et al., 1999). Conversely, Anabousi et al showed that incorporation of PEGylated lipids into the liposomes induced a steric stabilization with liposomes maintaining a spherical shape (Anabousi et al., 2005). AFM images of the surface of unconjugated liposomes were smooth and no structures could be observed, whereas conjugation of transferrin to the surface of PEGylated liposomes were visualized as small globular structures (Anabousi et al., 2005). Similarly, Moutardier et al manufactured liposomes with polymeric cores (LSP) that consisted of drugs loaded into polymeric particles that formed the core of lipid vesicles (Moutardier et al., 2003). Images taken using AFM showed the presence of a polymer network on the exterior surface, which suggested that the collagen polymeric core radiated out and formed a surface layer on the LSP (Moutardier et al., 2003). In addition to surface structural details, AFM can provide information about the mechanical and chemical properties of a sample surface through force measurements (Ruozi et al., 2007). For example, Mao et al. used this technique to assess the elasticity and adhesive properties of liposomes (Mao et al., 2004). One limitation of AFM is the need for nanoparticles to be adsorbed onto support surfaces, such as mica or silicon wafers. The adsorption of liposomes onto a solid substrate has the potential to modify the size and shape of the vesicles, and cause their flattening. (Ruozi et al., 2007). In addition, the periodic contact of the probing tip can drag the liposomes as it moves across the vesicles in a sample (Jass et al., 2000). Despite this, AFM is still a useful tool in the evaluation of liposomes.

CONCLUSION

There is a wide range of imaging techniques available for evaluating the morphology of liposomes, with each having its own advantages and disadvantages. Light microscopy can provide general details regarding the size and shape of larger vesicles and the homogeneity of a sample in a relatively fast manner. Conversely, TEM is the most commonly used technique to examine the morphology of liposomes in much more detail; however, the potential for structural changes with each TEM sub-type due to staining and/or exposure to vacuum conditions need to be considered when interpreting the results. ESEM is most useful when determining liposomal changes in response to the environment, whereas AFM is emerging as a useful method in the morphological analysis of nanoparticles and provides maximum resolution of the liposomal surface. Both AFM and TEM are capable of imaging ligands conjugated to the surface of liposomes and provide complementary information on surface modifications. Overall, the choice of technique is dependent on what morphological characteristics and degree of detail are required. In addition, understanding the potential effects of the sample preparation method of each imaging technique is important in the selection process.

AUTHOR CONTRIBUTIONS

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REFERENCES

Adler, K., and Schiemann, J. (1985). Characterization of liposomes by scanning electron microscopy and the freeze-fracture technique. *Micron Microsc. Acta* 16, 109–113. doi: 10.1016/0739-6260(85)90039-5

Almgren, M., Edwards, K., and Karlsson, G. (2000). Cryo transmission electron microscopy of liposomes and related structures. *Colloids Surf. A Physicochem. Eng. Asp.* 174, 3–21. doi: 10.1016/S0927-7757(00)00516-1

Anabousi, S., Laue, M., Lehr, C. M., Bakowsky, U., and Ehrhardt, C. (2005). Assessing transferrin modification of liposomes by atomic force microscopy and transmission electron microscopy. *Eur. J. Pharm. Biopharm.* 60, 295–303. doi: 10.1016/j.ejpb.2004.12.009

Bagatolli, L. A. (2009). *Membranes and Fluorescence Microscopy. Reviews in Fluorescence 2007*. New York, NY: Springer, 33–51. doi: 10.1007/978-0-387-88722-7_2

Bakker-Woudenberg, I. A., Storm, G., and Woodle, M. C. (1994). Liposomes in the treatment of infections. *J. Drug Target.* 2, 363–371. doi: 10.3109/10611869408996811

Bendas, G. (2001). Immunoliposomes: a promising approach to targeting cancer therapy. *BioDrugs* 15, 215–224. doi: 10.2165/00063030-200115040-00002

Bendas, G., Krause, A., Bakowsky, U., Vogel, J., and Rothe, U. (1999). Targetability of novel immunoliposomes prepared by a new antibody conjugation technique. *Int. J. Pharm.* 181, 79–93. doi: 10.1016/S0378-5173(99)00002-2

Bibi, S., Kaur, R., Henriksen-Lacey, M., McNeil, S. E., Wilkhu, J., Lattmann, E., et al. (2011). Microscopy imaging of liposomes: from coverslips to environmental SEM. *Int. J. Pharm.* 417, 138–150. doi: 10.1016/j.ijpharm.2010.12.021

Bouvrais, H., Pott, T., Bagatolli, L. A., Ipsen, J. H., and Meleard, P. (2010). Impact of membrane-anchored fluorescent probes on the mechanical properties of lipid bilayers. *Biochim. Biophys. Acta* 1798, 1333–1337. doi: 10.1016/j.bbamem.2010.03.026

Cullis, P. R., Chonn, A., and Semple, S. C. (1998). Interactions of liposomes and lipid-based carrier systems with blood proteins: relation to clearance behaviour in vivo. *Adv. Drug Deliv. Rev.* 32, 3–17. doi: 10.1016/S0169-409X(97)00128-2

Ding, B. S., Dziubla, T., Shuvaev, V. V., Muro, S., and Muzykantov, V. R. (2006). Advanced drug delivery systems that target the vascular endothelium. *Mol. Interv.* 6, 98–112. doi: 10.1124/mi.6.2.7

Henry, C. R. (2005). Morphology of supported nanoparticles. *Prog. Surf. Sci.* 80, 92–116. doi: 10.1016/j.progsurf.2005.09.004

Hua, S. (2013). Targeting sites of inflammation: intercellular adhesion molecule-1 as a target for novel inflammatory therapies. *Front. Pharmacol.* 4:127. doi: 10.3389/fphar.2013.00127

Hua, S., Marks, E., Schneider, J. J., and Keely, S. (2015). Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: selective targeting to diseased versus healthy tissue. *Nanomedicine* 11, 1117–1132. doi: 10.1016/j.nano.2015.02.018

Hua, S., and Wu, S. Y. (2013). The use of lipid-based nanocarriers for targeted pain therapies. *Front. Pharmacol.* 4:143. doi: 10.3389/fphar.2013.00143

Ishida, T., Harashima, H., and Kiwada, H. (2001). Interactions of liposomes with cells in vitro and in vivo: opsonins and receptors. *Curr. Drug Metab.* 2, 397–409. doi: 10.2174/1389200013338306

Jass, J., Tjarnhage, T., and Puu, G. (2000). From liposomes to supported, planar bilayer structures on hydrophilic and hydrophobic surfaces: an atomic force microscopy study. *Biophys. J.* 79, 3153–3163. doi: 10.1016/S0006-3495(00)76549-0

Klymchenko, A. S., Oncul, S., Didier, P., Schaub, E., Bagatolli, L., Duportail, G., et al. (2009). Visualization of lipid domains in giant unilamellar vesicles using an environment-sensitive membrane probe based on 3-hydroxyflavone. *Biochim. Biophys. Acta* 1788, 495–499. doi: 10.1016/j.bbamem.2008.10.019

Kuntsche, J., Horst, J. C., and Bunjes, H. (2011). Cryogenic transmission electron microscopy (cryo-TEM) for studying the morphology of colloidal drug delivery systems. *Int. J. Pharm.* 417, 120–137. doi: 10.1016/j.ijpharm.2011.02.001

Le Bihan, O., Bonnafous, P., Marak, L., Bickel, T., Trepout, S., Mornet, S., et al. (2009). Cryo-electron tomography of nanoparticle transmigration into liposome. *J. Struct. Biol.* 168, 419–425. doi: 10.1016/j.jsb.2009.07.006

Liang, X., Mao, G., and Ng, K. Y. (2004a). Mechanical properties and stability measurement of cholesterol-containing liposome on mica by atomic force microscopy. *J. Colloid Interface Sci.* 278, 53–62.

Liang, X., Mao, G., and Ng, K. Y. (2005). Effect of chain lengths of PEO-PPO-PEO on small unilamellar liposome morphology and stability: an AFM investigation. *J. Colloid Interface Sci.* 285, 360–372. doi: 10.1016/j.jcis.2004.11.042

Liang, X., Mao, G., and Simon Ng, K. Y. (2004b). Probing small unilamellar EggPC vesicles on mica surface by atomic force microscopy. *Colloids Surf. B Biointerfaces* 34, 41–51.

Mao, G., Liang, X., and Ng, K. Y. S. (2004). *Encyclopedia of Nanoscience and Nanotechnology*. New York, NY: Marcel Dekker.

Mastrobattista, E., Koning, G. A., and Storm, G. (1999). Immunoliposomes for the targeted delivery of antitumor drugs. *Adv. Drug Deliv. Rev.* 40, 103–127. doi: 10.1016/S0169-409X(99)00043-5

Mertins, O., and Dimova, R. (2013). Insights on the interactions of chitosan with phospholipid vesicles. Part II: membrane stiffening and pore formation. *Langmuir* 29, 14552–14559. doi: 10.1021/la4032199

Metselaar, J. M., and Storm, G. (2005). Liposomes in the treatment of inflammatory disorders. *Expert Opin. Drug Deliv.* 2, 465–476. doi: 10.1517/17425247.2.3.465

Mohammed, A. R., Weston, N., Coombes, A. G., Fitzgerald, M., and Perrie, Y. (2004). Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. *Int. J. Pharm.* 285, 23–34. doi: 10.1016/j.ijpharm.2004.07.010

Moutardier, V., Tosini, F., Vlieghe, P., Cara, L., Delpero, J. R., and Clerc, T. (2003). Colloidal anticancer drugs bioavailabilities in oral administration models. *Int. J. Pharm.* 260, 23–38. doi: 10.1016/S0378-5173(03)00231-X

Murphy, D. B., and Davidson, M. W. (2012a). *Confocal Laser Scanning Microscopy. Fundamentals of Light Microscopy and Electronic Imaging*, 2nd Edn. Hoboken, NJ: John Wiley & Sons, 265–305. doi: 10.1002/9781118382905.ch13

Murphy, D. B., and Davidson, M. W. (2012b). *Fluorescence Microscopy. Fundamentals of Light Microscopy and Electronic Imaging*. Weinheim: John Wiley & Sons, 199–231. doi: 10.1002/9781118382905.ch11

Murphy, D. B., and Davidson, M. W. (2012c). *Fundamentals of Light Microscopy and Electronic Imaging*. Hoboken, NJ: John Wiley & Sons, 1–19. doi: 10.1002/9781118382905

Murphy, D. B., and Davidson, M. W. (2012d). *Polarization Microscopy. Fundamentals of Light Microscopy and Electronic Imaging*. Hoboken, NJ: John Wiley & Sons, 153–171. doi: 10.1002/9781118382905.ch9

Muscariello, L., Rosso, F., Marino, G., Giordano, A., Barbarisi, M., Cafiero, G., et al. (2005). A critical overview of ESEM applications in the biological field. *J. Cell. Physiol.* 205, 328–334. doi: 10.1002/jcp.20444

Nallamothu, R., Wood, G. C., Pattillo, C. B., Scott, R. C., Kiani, M. F., Moore, B. M., et al. (2006). A tumor vasculature targeted liposome delivery system for combretastatin A4: design, characterization, and in vitro evaluation. *AAPS PharmSciTech* 7:E32. doi: 10.1208/pt070232

Nirale, N. M., Vidhate, R. D., and Nagarsenker, M. S. (2009). Fluticasone propionate liposomes for pulmonary delivery. *Indian J. Pharm. Sci.* 71, 709–711.

Orlova, E. V., Sherman, M. B., Chiu, W., Mowri, H., Smith, L. C., and Gotto, A. M. Jr. (1999). Three-dimensional structure of low density lipoproteins by electron cryomicroscopy. *Proc. Natl. Acad. Sci. U.S.A.* 96, 8420–8425. doi: 10.1073/pnas.96.15.8420

Ruozi, B., Belletti, D., Tombesi, A., Tosi, G., Bondioli, L., Forni, F., et al. (2011). AFM, ESEM, TEM, and CLSM in liposomal characterization: a comparative study. *Int. J. Nanomedicine* 6, 557–563. doi: 10.2147/IJN.S14615

Ruozi, B., Tosi, G., Forni, F., Fresta, M., and Vandelli, M. A. (2005). Atomic force microscopy and photon correlation spectroscopy: two techniques for rapid characterization of liposomes. *Eur. J. Pharm. Sci.* 25, 81–89. doi: 10.1016/j.ejps.2005.01.020

Ruozi, B., Tosi, G., Leo, E., and Vandelli, M. A. (2007). Application of atomic force microscopy to characterize liposomes as drug and gene carriers. *Talanta* 73, 12–22. doi: 10.1016/j.talanta.2007.03.031

Ruozi, B., Tosi, G., Tonelli, M., Bondioli, L., Mucci, A., Forni, F., et al. (2009). AFM phase imaging of soft-hydrated samples: a versatile tool to complete the chemical-physical study of liposomes. *J. Liposome Res.* 19, 59–67. doi: 10.1080/08982100802584071

Sawant, R. R., and Torchilin, V. P. (2012). Challenges in development of targeted liposomal therapeutics. *AAPS J.* 14, 303–315. doi: 10.1208/s12248-012-9330-0

Senior, J. H. (1987). Fate and behavior of liposomes in vivo: a review of controlling factors. *Crit. Rev. Ther. Drug Carrier Syst.* 3, 123–193.

Sercombe, L., Veerati, T., Moheimani, F., Wu, S. Y., Sood, A. K., and Hua, S. (2015). Advances and challenges of liposome assisted drug delivery. *Front. Pharmacol.* 6:286. doi: 10.3389/fphar.2015.00286

Severs, N. J. (2007). Freeze-fracture electron microscopy. *Nat. Protoc.* 2, 547–576. doi: 10.1038/nprot.2007.55

Sitterberg, J., Ozcetin, A., Ehrhardt, C., and Bakowsky, U. (2010). Utilising atomic force microscopy for the characterisation of nanoscale drug delivery systems. *Eur. J. Pharm. Biopharm.* 74, 2–13. doi: 10.1016/j.ejpb.2009.09.005

Torchilin, V. P. (1994). Immunoliposomes and PEGylated immunoliposomes: possible use for targeted delivery of imaging agents. *Immunomethods* 4, 244–258. doi: 10.1006/immu.1994.1027

Van Antwerpen, R., and Gilkey, J. C. (1994). Cryo-electron microscopy reveals human low density lipoprotein substructure. *J. Lipid Res.* 35, 2223–2231.

Wang, X., Song, Y., Su, Y., Tian, Q., Li, B., Quan, J., et al. (2015). Are PEGylated liposomes better than conventional liposomes? A special case for vincristine. *Drug Deliv.* 29, 1–9. doi: 10.3109/10717544.2015.1027015

Weisman, S., Hirsch-Lerner, D., Barenholz, Y., and Talmon, Y. (2004). Nanostructure of cationic lipid-oligonucleotide complexes. *Biophys. J.* 87, 609–614. doi: 10.1529/biophysj.103.033480

Willis, M., and Forssen, E. (1998). Ligand-targeted liposomes. *Adv. Drug Deliv. Rev.* 29, 249–271. doi: 10.1016/S0169-409X(97)00083-5

Zununi Vahed, S., Salehi, R., Davaran, S., and Sharifi, S. (2017). Liposome-based drug co-delivery systems in cancer cells. *Mater. Sci. Eng. C Mater. Biol. Appl.* 71, 1327–1341. doi: 10.1016/j.msec.2016.11.073

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MINI REVIEW
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Risk Assessment and Risk Minimization in Nanomedicine: A Need for Predictive, Alternative, and 3Rs Strategies

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The use of nanomaterials in medicine has grown very rapidly, leading to a concern about possible health risks. Surely, the application of nanotechnology in medicine has many significant potentialities as it can improve human health in at least three different ways: by contributing to early disease diagnosis, improved treatment outcomes and containment of health care costs. However, toxicology or safety assessment is an integral part of any new medical technology and the nanotechnologies are no exception. The principle aim of nanosafety studies in this frame is to enable safer design of nanomedicines. The most urgent need is finding and validating novel approaches able to extrapolate acute *in vitro* results for the prediction of chronic *in vivo* effects and to this purpose a few European initiatives have been launched. While a "safe-by-design" process may be considered as utopic, "safer-by-design" is probably a reachable goal in the field of nanomedicine.

Keywords: nanomaterial, nanomedicine, nanosafety, risk assessment, risk minimization

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INTRODUCTION

Nanomedicine encloses many potential promises, ranging from optimized, targeted, and even personalized treatments with decreased toxicity, to very sensitive, and cheaper diagnostic approaches with contained costs, innovative functionalized biomaterials, and the prospect of cellular and tissue regeneration strategies (Wagner et al., 2006; Senjen, 2013).

Nanotechnology-based approaches have already been translated into highly accurate and sensitive diagnostic tests, most prominent of which is the early detection of neoplastic disease (Ferrari et al., 2009), targeted therapeutic interventions, following the statement that "targeted delivery will revolutionize disease treatment" (Duncan and Gaspar, 2011) and theranostic applications, having the possibility to combine in the same disease intervention both diagnosis (through imaging) and treatment (through thermal ablation) (Lammers et al., 2011).

Nanotechnological devices used in nanomedicine possess unique properties, not found in identical devices of smaller or larger dimensions, as they stem from their nanoscale dimension (Ferrari et al., 2009). However, a consensus has not been reached yet as for a scientific definition of "nanoparticle" (NP) (Satalkar et al., 2016) and as a consequence slightly different size limit definitions are applied in different fields of nanotechnology (Schütz et al., 2013).

In this review we first summarize the current state regarding safety evaluation of nano-based therapeutics and then we focus on the growing need for nanosafety studies for safer design of

nanomedicines, including the employment of novel acute *in vitro* studies to extrapolate chronic effects that occur *in vivo*.

RISK ASSESSMENT AND RISK MINIMIZATION IN NANOMEDICINE

The implementation of nanotechnology in medicine is a process that has occurred rapidly, suddenly moving from basic research and laboratory experimentation to clinical trials (Kola and Landis, 2004; Etheridge et al., 2013; Hafner et al., 2014). Many nanomedicine formulations have already entered the market. A recent study identified 247 nanomedicine applications and products, a very significant number, approved for or nearing in-human use (Etheridge et al., 2013) and it has been envisioned that the worldwide nanomedicine market may double by 2019 (BBCResearch, 2015). In the majority of cases, these artifacts are nanoformulations of current or novel drugs (58%) or nanobiomaterials (25%); however, nanotechnology has the potential to add innovative functionality to many pharmaceutical products and medical devices (Wagner et al., 2006). As for the regulatory aspects, it remains to be determined if nanomedicines fall into the category of medicinal products or medical devices. EU legislation makes clear distinction between the two, resulting in different regulatory approaches for risk assessment of medicinal products and medical devices. In the case of medicinal products, suitable clinical trials have to be performed prior to provision of a preliminary market authorisation for human use (Directive 2001/83/EC), while in the case of medical device market introduction requires lesser degrees of testing that can vary on the basis of the risk category the device falls into [Directive 2007/47/EC currently under revision: COM (2012) 542 final].

An elevated degree of unpredictability about prospective hazards and true advantages of NPs and nanomedicines, however, created remarkable obstacles along this translational pathway (Resnik and Tinkle, 2007). As such, nanotechnological approaches have opened up a few issues with respect to their proper risk assessment and risk minimization (Hogle, 2012), with particular emphasis on human and environmental toxicity (Allhoff, 2009; Ramachandran et al., 2012). Especially compelling in this respect are "first in human" (FIH) trials of nanotechnology medical applications, as they raise the highest degree of unpredictability in all clinical area (Kimmelman and John London, 2011). Especially important for FIH nanomedicine trials is the explicit description of the study purposes within the consent form. Today, the primary and sometimes exclusive aim of FIH trials is safety (King, 2012), even though innovative technologies like nanomedicines do not always fit the classical clinical trial phases that are followed when a new drug is developed. Thus, the information contained in the consent form should focus on these purposes and emphasize all aspects related to safety testing and risk identification (King, 2012).

Research in the field of nanomaterials (NMs) applied to medicine has continued to grow upon time, but it has been primarily focused around technological improvement, and not directed toward the definition of the potential risks of nanoproducts, thus nanosafety is an area that has remained poorly assessed. The fate of a nanomaterial upon entrance in the organism, whether it will be accumulated and become toxic, or rendered available at a biological level and transformed, or if and how it will interact with cells and macromolecules inside cells, are all crucial aspects that need to be understood. The conventional approaches listed in current test guidelines are not very likely to turn out as appropriate for the assessment of nanomedicine risks, rendering it urgent to develop NM-specific standards, guidelines, and tools. It is well-known, in fact, that often the bulk materials behave different in the nanometer regime and there is the need of generally accepted test methods for the characterization of nanomaterials. The methods currently existing can be not at all suitable to characterize nanomaterials for their specific properties. For example, when nanoparticles are dispersed in water, air or biological media they show a tendency to agglomerate and can even lose their nano-dimension. For this, it is important a careful and wide morphological, physico-chemical, and *in vitro* and *in vivo* biological characterization not only on the manufactured nanomaterials but also on nanomaterials after contact with relevant media. The evaluation of the effects of dispersion methods and of molecular interaction with biological components, cells and tissues on properties of nanomaterials represent a fundamental step for an effective control of nanomaterial risk. The degradation of the nanomaterials in the biological environment, the release of molecules or debris and the functionalization with organic substances could induce cytotoxic effects to be explored using methods already employed and validated in nanotoxicology but also improving the physico-chemical characterization. *QualityNano* (www.qualitynano.eu; finished in 2015) has represented one of the first European initiatives along this line. It was an analytical research infrastructure addressing quality in NM safety assessment, through driving reliable and reproducible approaches to nanometrology and NM characterization before, during and after exposure to living systems. It included the development of standard operating protocols (SOPs) for analysis of the possible risks posed by NMs, focusing on assay reproducibility, use of appropriate positive and negative controls and controlling dose delivered to living systems (Senjen, 2013).

Assessment and management of risks, as well as risk communication are among the most challenging issues for nanomedicine clinical research (Resnik and Tinkle, 2007). Surely, our understanding and knowledge of different nanosized materials can be improved by single *in vivo* animal experiments and *ex vivo* laboratory testing, yet when a new nanomedicine product is to be tested in Phase I clinical trial they are not sufficient to resolve all of the uncertainty surrounding the first exposure of a human subject. What can be ethically accepted is that the risks potentially posed to human subjects by the new therapy are reasonable in relation to the new therapy potential benefits and, from a regulatory standpoint, that risks to the subject and society are minimized, wherever possible (Emanuel et al., 2000).

PREDICTIVE TOXICOLOGICAL APPROACHES, ALTERNATIVE TEST STRATEGIES, AND 3RS APPROACHES

Extensive preclinical and clinical testing is needed prior to application of nanomedicine products in the three relevant areas of diagnosis, prevention, and treatment of disease, yet many aspects of NMs, including their toxicological, pharmacological, and immunological properties, have entered the scientific exploration only recently. Early safety studies are exceedingly needed to define whether the risk to benefit displayed by a specific nanomedicine is acceptable for the proposed use, thus determining if that nanotechnology will have the promise for further development in a clinical application (Duncan and Gaspar, 2011). Importantly, traditional approaches to toxicology, which are inherently descriptive in nature, will need to shift to predictive toxicology and this shift ascribes to both chemicals in general and NMs in particular (Oberdorster, 2010).

- Predictive toxicology is based on mechanism-based approaches relying on high-throughput screening (HTS) techniques. (i) The starting point is the generation of *in vitro* toxicity data resulting from the application of multiparametric, automated screening procedures. This *in vitro* phase of work may predict the possibility for disease or other pathological outcomes *in vivo,* based on the specific physicochemical properties of engineered NMs that are described (Nel et al., 2013b). (ii) The *in vivo* step will first of all validate the HTS techniques and will then improve them by establishing clear structure activity relationships. (iii) Then heat maps are developed on the basis of normalized data set, and self-organizing map features are exploited to organize all these information. (iv) Finally, appropriate combinations of both *in vitro* and *in vivo* approaches can be defined, with the final goal to establish hazard ranking and modeling. The landmark 2007 report from the US National Academy of Sciences, "Toxicity Testing in the Twenty-first Century: A Vision and a Strategy" (http://www.nap.edu/catalog.php?record_id=11970) is in agreement with this operation modality, clearly defining that a transition from qualitative laboratory testing and descriptive animal studies to mechanistic, quantitative testing funded on the employment of human cell types and high-throughput approaches will dramatically increase efficiency of toxicity evaluation (Nel et al., 2013b). Hazard assessment of large numbers of NMs can in this way be performed using pathways of toxicity (POTs), consisting in mechanism-based testing and representing the aim of the predictive toxicology approach (Nel, 2013). For the successful implementation of this methodology, careful selection of HTS techniques to be used *in vitro* as well as of POTs designed at the cellular level is needed, in order for them to reflect as many as possible pathogenic effects at the organism level. Although many methods and protocols were developed and validated for a predictive risk assessment of NMs, more work is needed regarding both physicochemical properties of NMs and their interaction with biological media. For example, the surface chemistry of the particles should be evaluated with particular attention considering that dissolution/dispersion and fate of NMs in biological media are affected by particle surface, surface charge, and radical formation potential.
- Use of alternative test strategies (ATS), aimed at reducing the number of animal testing by widening the employment of *in vitro* and *in silico* strategies, represents a promising new toxicological paradigm for NMs in medicine (Nel, 2013). Existing and emerging methods used as part of an ATS are presented in **Table 1**. HTS techniques, high-content screening, and computational modeling are all important resources for ATS, having the potentiality to analyse in a comparative way many NMs simultaneously (Nel, 2013). Further, the use of ATS approach allows for multiple hazard assessment steps during the entire process of product development and provides large enough amounts of data to reduce the number of animals used by prioritizing testing at each of the incremental assessment stages described above (Nel et al., 2013a). As a matter of fact, numerous challenges still need to be faced prior to complete acceptance of ATS. For example, cellular HTS is still limited when a chronic disease condition is to be studied through predictive toxicological approaches, because currently *in vitro* cultured cells cannot recapitulate the chronology of the multistep process leading to a chronic disease at the organ or systemic level (Leist and Hartung, 2013). It is also not always easy to discriminate between end-points that disclose adverse outcomes, or that may conduct to adverse outcomes later on, and those that reflect non-adverse outcomes (Slikker et al., 2004). For regulatory purposes, risk assessment or risk management based on the use of ATS to replace for animal testing is not yet at the level of general acceptance. However, the potential utility of ATS approaches to investigate NM hazard is not under discussion and there is a general agreement about the application of ATS approaches to prioritize NMs for further subsequent toxicity testing and risk assessment prior to or upon product development (Nel et al., 2013a). Future strategies should include refinement of existing tests, such as development of organotypic 3D co-cultures, use of primary cells or stem-cell-derived systems and expansion of endpoints (i.e., carcinogenicity for chronic exposure) and their simultaneous testing. In addition, there is a need to predict the distribution, translocation, and bioaccumulation of NMs throughout the human body after exposure: pharmacokinetic (PBPK) models already developed in animal for specific NM are expected to be extrapolated also to humans. Finally, a comparison between *in vitro* and *in vivo* tests and long-term evaluation should be carefully considered.
- Reduction of *in vivo* experiments through the employment of alternative testing approaches is in agreement with the 3Rs rule (refine, reduce, and replace animal testing). There is an opportunity to improve regulatory toxicology by first optimizing the use of the entire amount of existing information concerning groups of structurally similar materials, by second gaining information from *in vitro* and *in silico* experimental approaches, and finally conducting targeted animal testing only when necessary (Hartung, 2009). Ideally, these types of strategies should encompass decision

TABLE 1 | Existing and emerging methods used as part of an ATS for NM evaluation.

Test type	Aim	Key stages or assay	Limitation	Ref
Genotoxicity	Rapid measurement of DNA damage, chromosomal damage; detection of upregulated DNA damage signaling pathways	- MN (Micronucleus assay) - CometChip Platform - γ-H2AX assay - FADU (Fluorimetric Detection of Alkaline DNA Unwinding) assay - ToxTracker reporter assay	Many factors can artificially influence assay results, as material and environment	Nelson et al., 2017
QSAR (Quantitative Structure Activity Relationships)	Prediction of nanomaterial exposure-dose-response	Steps of data assembling, structure characterization, model construction, model evaluation, and lastly interpretation of mechanisms	Small number of data sets	Winkler, 2016
SSDs (species sensitivity distributions)	Estimation of the maximum acceptable concentrations of chemicals in environmental risk assessment	Computational approaches	Few data known	Chen et al., 2017
Band gap analysis	Prediction of toxic potential using metal oxide conduction band energy levels	*In vitro* toxicological effect related to conduction energy and metal dissolution	Limited to metal based nanomaterials	Zhang et al., 2012
Cytotoxicity	Screening of nanomaterial-induced cytotoxicity	- Cellular metabolic activity - Oxidative stress - Apoptosis - Cell membrane integrity - Impedance based integrity	Time-consuming, labor-intensive, complex, and in some instances unreliable owing to NM interferences	Cimpan et al., 2013; Guadagnini et al., 2015; Accomasso et al., 2016
OMICS	Identification of new pathways and mechanisms in nanotoxicity not visible in conventional testing	1.1.1 Epigenomics—miRNomics 1.1.2 Epigenomics—DNA methylation and histone modification 1.1.3 Transcriptomics 1.1.4 Proteomics 1.1.5 Metabolomics	Request for high sample quality (freezing, protection against degradation) Lack of standardization of sample preparation Predictive value of the omics techniques not entirely clear	Fröhlich, 2017
High-content analysis	Capacity for monitoring a range of morphometric, functional, and biochemical properties of cells	Simultaneous identification of different parameters using fluorescence	Possible fluorescence-dye toxicity Limitation in adequate cell line	Brayden et al., 2015

points that depend on *ad interim* results and a critical aspect of the validation process becomes a correlation between *in vitro* and *in silico* with *in vivo* results. From an ethical and economical perspective it is not acceptable to test each NM in animals, thus a triage step based on an *in vitro* screening of these materials is necessary. Efforts in this direction are being made in the USA (Nel, 2013), but also in Europe collaborative initiatives are being created to increase open conveyance and sharing of results between different research groups. Overall, testing programme for regulatory purpose addressed a series of physico-chemical endpoints including methods and assays for a list of manufactured NMs, mostly inorganic carbon and metal oxide NMs (TiO_2, SiO_2, ZnO, CeO_2). For different physico-chemical endpoints, several methods were used to evaluate: chemical composition (assay: EDX, ICP-OES ICP-MS, CHN elemental analysis), size [assay: DLS, TEM, SEM, AFM, (U)SAXS), WAXS], shape (TEM), coating (XPS, STEM-EDS, FTIR analysis of functional groups), surface area (VSSA, SAXS), Water solubility/dispersibility, cristallite size (XRD) (Rasmussen et al., 2018). *In vitro* studies addressed cytotoxicity, immunotoxicity, and genotoxicity testing using different cell culture models (i.e., blood, lung, placenta, brain, liver, gastrointestinal system) according to harmonized protocols.

The *NanoTEST* project (http://www.nanotest-fp7.eu/; finished in 2012) was one of the first examples. Many efforts were put in defining appropriate standard protocols, whose frequent lack represented an important problem experienced in testing NP potential hazards before clinical application (Juillerat-Jeanneret et al., 2015). A representative selection of commercial NMs currently or soon-to-be-applied in human medicine was investigated. To identify relevant short-term hazard models, the project used several standard toxicity assays for different markers such as cell viability, pro inflammatory response, oxidative stress, genotoxicity, immunotoxicity, cell uptake, and transport. Upon completion of the study, indications for full appraisal of NP toxicity included a few cytotoxicity measurements, a set of 2–3 representative cell types and five NP concentrations (Dusinska et al., 2015). *NANoREG* (www.nanoreg.eu; finished in 2017), *FutureNanoNeeds* (www.futurenanoneeds.eu; finished in 2017), and the ongoing *NanoReg2* (www.nanoreg2.eu) are three other European projects aimed at defining a customary European strategy to the regulatory testing of fabricated NMs and at evolving an innovative frame to allow proper classification, better naming as well as hazard and environmental impact assessment of the future NMs before their extensive industrial employment.

APPLICATION OF ALTERNATIVE TEST STRATEGIES TO RISK ASSESSMENT AND MINIMIZATION IN NANOMEDICINE

A number of different nano-specific, well-designed ATS are under development having the potentiality to provide answers to focused NM toxicity questions (Shatkin and Ong, 2016).

When the NM under study has an unknown toxicity, adoption of a Weight of Evidence (WoE) approach can be considered. Risk assessment is determined following careful hazard identification and prioritization taking into consideration and weighting all *in vitro* data available, both qualitative and quantitative, even in the absence of animal data. WoE mainly represents a methodological approach, where a collection of studies is analyzed based on expert opinions, systematic reviews or meta-analyses. When possible, quantitative WoE evaluations are also applied (Hristozov et al., 2014), for instance for prioritizing the riskiest occupational exposure scenarios that, in the case of NMs, can include processing methods, handling methods, length of time of exposure, protective equipment. They will be all considered and weighted accordingly, on the basis of quantitative data and expert judgement. Multiple techniques to test one end point should be applied to generate data with a high enough quality to allow for regulatory decisions to be taken based on WoE approaches.

Intelligent/Integrated Testing Strategies (ITS) are applied for the identification and prioritization of nanosafety research needs (Stone et al., 2014). Through this method, hypothesis-driven questions put to make risk decisions are answered by combining existing data, available analytical tools, experimental tests where the main goal is to avoid the need to test each developed NM. This strategy has been applied to accelerate the risk assessment process for materials of concern (Jaworska and Hoffmann, 2010) and benefits include reduced testing, consequently lowering the costs and limiting animal use; possibility to categorize NMs by (potential) mechanisms of action; applicability to a large assortment of testing strategies. Generally, ITS is based on of a stepped framework beginning with (i) an evaluation of existing data, that are organized using implements such as adverse outcome pathways (see below); (ii) measurement of chemical properties; (iii) biokinetic study of the NM; (iv) choice of suitable toxicity tests; and (v) employment of a WoE analysis that takes in consideration all the above results. Refinement steps can follow concerning both strategy and methods after which WoE is reevaluated (Oomen et al., 2014).

Conceptual frameworks such as adverse outcome pathways (AOP) are also been developed. Risk assessment is here performed through a sequential chain of events that are all causally linked and lead to an adverse outcome. Based on existing data, an initial molecular event is described and linked to a series of downstream key events acting at different biological levels (organism-cell-molecule) and eventually leading to the adverse outcome (Ankley et al., 2010).

All the above-mentioned approaches are needed in order to enable alignment of nanotoxicology with the 3Rs (Burden et al., 2017). The first step, establishment of a regulatory framework to enable implementation of alternative non-animal methods into risk assessment and acceptance, can benefit from WoE approaches to consider all available evidence from different non-animal methods. This will increase regulatory confidence in results from non-traditional methods, via guidelines and appropriate training, and will support risk assessors to understand the relevance and applicability of *in vitro* data for risk assessment and to adopt a rationale to deal with uncertainties and limitations inherent to experimental models (both *in vitro* and *in vivo*). The subsequent hazard prediction step will rely on ITS and adoption of a dual approach: hypothesis driven studies which test if a particular nanomaterial property impacts on toxicity, and studies which compare the toxicity of panels of nanomaterials. These parallel approaches will aim to accurately identify which NM properties confer toxicity and to establish a "reference data" for different endpoints for NMs which are deemed "representative" (dependent on the NM being studied) and the use of appropriate positive controls to relate the effects *in vitro/in vivo*. This involves ensuring that knowledge already in existence in other areas of toxicology is utilized to build knowledge within the discipline of nanotoxicology. After a validation step, AOPs frameworks can be exploited to adapt current standard *in vitro* approaches and to improve test item preparation, dosing, and understanding of toxicity mechanisms.

A rational design of the nanomaterials from the early phase of material selection, production method optimization, and product purification has to be considered of fundamental importance to prevent the safety issues of nanomaterial and increase their applicative potential. The concept of safer-by-design emphasizes the importance of the contribution of more scientists such as engineers, chemists, physicians, and biologists to contrast the challenges of nanomaterials and satisfy the needs of the EU to regulate manufactured nanomaterials. The use of advanced analytical techniques (i.e., ICP, AFM, Chemical Imaging, biomarker detection) or their combination for the study of interactions between nanomaterials-relevant media in parallel with a better control of the preparation process will likely open up new scenarios in nanotoxicology testing (Dusinska et al., 2017; Oomen et al., 2018).

CONCLUSIONS AND REMARKS

In nanomedicine, a proper risk evaluation in relation to health is unavoidable, in order to safeguard societal, ethical and regulatory acceptance, and public confidence. However, the individual testing approaches are limited and have turned out to be inadequate for nanotoxicology evaluations, thus risk assessment has needed to evolve to accommodate predictive toxicological analyses and ATS. The main short- to medium-term objectives should include an improved comprehension of processes of interaction of NMs utilized in nanomedicine with organs, tissues, and cells and a clear strategy to tackle critical topics connecting to toxicity assessment specifically with respect to alternatives to tests on animals. Among the most compelling future objectives will be the need to test nanomedicines not only in healthy physiological environments,

but also in disease environments that may alter biological responses and impact safety; in addition, to test nanoparticles not only individually but within complex mixtures, considering that nanoparticle incorporation into a variety of already utilized medical applications are likely to alter their risk profiles.

A safer-by-design concept has become increasingly important in risk assessment and minimization of nanomedicines, with the idea of integrating knowledge of NMs' potential adverse effects into the process of designing nanoproducts. This entails that nanomedicine safety is to be considered as an integrated route from the very first phases of research and innovation to the last phases of product validation, clearly different from the classical safety evaluation paradigm seeking to address potential concerns and to regulate NMs downstream, close to full product development, and market entrance.

AUTHOR CONTRIBUTIONS

CC and CG contributed conception and design of the study; CG wrote the first draft of the manuscript; LA and CC wrote sections of the manuscript; LA contributed design of the table. All authors read and approved the submitted version.

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REFERENCES

Accomasso, L., Gallina, C., Turinetto, V., and Giachino, C. (2016). Stem cell tracking with nanoparticles for regenerative medicine purposes: an overview. *Stem Cells Int.* 2016:7920358. doi: 10.1155/2016/7920358.

Allhoff, F. (2009). The coming era of nano medicine. *Am. J. Bioeth.* 9, 3–11. doi: 10.1080/15265160902985027

Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., et al. (2010). Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem.* 29, 730–741. doi: 10.1002/etc.34

BBCResearch (2015). N*anotechnology in Medical Applications: The Global Market*. Available online at: http://www.bccresearch.com/market-research/healthcare/nanotechnology-medical-applications-market-hlc069c.html.

Brayden, D. J., Cryan, S. A., Dawson, K. A., O'Brien, P. J., and Simpson, J. C. (2015). High-content analysis for drug delivery and nanoparticle applications. *Drug Discov. Today* 20, 942–957. doi: 10.1016/j.drudis.2015.04.001

Burden, N., Aschbergerb, K., Chaudhry, Q., Clift, M. J. D., Fowler, P., Johnston, H., et al. (2017). Aligning nanotoxicology with the 3Rs: what is needed to realise the short, medium and long-term opportunities? *Regul. Toxicol. Pharmacol.* 91, 257–266. doi: 10.1016/j.yrtph.2017.10.021

Chen, G., Peijnenburg, W., Xiao, Y., and Vijver, M. G. (2017). Current knowledge on the use of computational toxicology in hazard assessment of metallic engineered nanomaterials. *Int. J. Mol. Sci.* 18:E1504. doi: 10.3390/ijms18071504.

Cimpan, M. R., Mordal, T., Schölermann, J., Allouni, Z. E., Pliquett, U., and Cimpan, E. (2013). An impedance-based high-throughput method for evaluating the cytotoxicity of nanoparticles. *J. Phys.* 429:012026. doi: 10.1088/1742-6596/429/1/012026

Duncan, R., and Gaspar, R. (2011). Nanomedicine(s) under the microscope. *Mol. Pharm.* 8, 2101–2141. doi: 10.1021/mp200394t

Dusinska, M., Boland, S., Saunders, M., Juillerat-Jeanneret, L., Tran, L., Pojana, G., et al. (2015). Towards an alternative testing strategy for nanomaterials used in nanomedicine: lessons from NanoTEST. *Nanotoxicology* 9(Suppl. 1), 118–132. doi: 10.3109/17435390.2014.991431

Dusinska, M., Tulinska, J., El Yamani, N., Kuricova, M., Liskova, A., Rollerova, E., et al. (2017). Immunotoxicity, genotoxicity and epigenetic toxicity of nanomaterials: new strategies for toxicity testing? *Food Chem. Toxicol.* 109:797–811. doi: 10.1016/j.fct.2017.08.030

Emanuel, E. J., Wendler, D., and Grady, C. (2000). What makes clinical research ethical? *JAMA* 283, 2701–2711. doi: 10.1001/jama.283.20.2701

Etheridge, M. L., Campbell, S. A., Erdman, A. G., Haynes, C. L., Wolf, S. M., and McCullough, J. (2013). The big picture on nanomedicine: the state of investigational and approved nanomedicine products. *Nanomedicine* 9, 1–14. doi: 10.1016/j.nano.2012.05.013

Ferrari, M., Philibert, M. A., and Sanhai, W. R. (2009). Nanomedicine and society. *Clin. Pharmacol. Ther.* 85, 466–467. doi: 10.1038/clpt.2008.276

Fröhlich, E. (2017). Role of omics techniques in the toxicity testing of nanoparticles *J. Nanobiotechnol.* 15:84. doi: 10.1186/s12951-017-0320-3

Guadagnini, R., Moreau, K., Hussain, S., Marano, F., and Boland, S. (2015). Toxicity evaluation of engineered nanoparticles for medical applications using pulmonary epithelial cells. *Nanotoxicology* 9(Suppl. 1), 25–32. doi: 10.3109/17435390.2013.855830.

Hafner, A., Lovrić, J., Lakoš, G. P., and Pepić, I. (2014). Nanotherapeutics in the EU: an overview on current state and future directions. *Int. J. Nanomedicine* 9, 1005–1023. doi: 10.2147/IJN.S55359

Hartung, T. (2009). Toxicology for the twenty-first century. *Nature* 460, 208–212. doi: 10.1038/460208a

Hogle, L. F. (2012). Concepts of risk in nanomedicine research. *J. Law Med. Ethics* 40, 809–822. doi: 10.1111/j.1748-720X.2012.00709.x

Hristozov, D. R., Zabeo, A., Foran, C., Isigonis, P., Critto, A., Marcomini et al. (2014). A weight of evidence approach for hazard screening of engineered nanomaterials. *Nanotoxicology* 8, 72–87. doi: 10.3109/17435390.2012.750695

Jaworska, J., and Hoffmann, S. (2010). Integrated testing strategy (ITS)—Opportunities to better use existing data and guide future testing in toxicology. *ALTEX* 27, 231–242. doi: 10.14573/altex.2010.4.231

Juillerat-Jeanneret, L., Dusinska, M., Fjellsbø L. M., Collins, A. R., Handy, R. D., Riediker, M., et al. (2015). Biological impact assessment of nanomaterial used in nanomedicine. introduction to the NanoTEST project. *Nanotoxicology* 9(Suppl. 1), 5–12. doi: 10.3109/17435390.2013.826743

Kimmelman, J., and John London, A. (2011). Predicting harms and benefits in translational trials: ethics, evidence, and uncertainty. *PLoS Med.* 8:e1001010. doi: 10.1371/journal.pmed.1001010

King, N. M. (2012). Nanomedicine first-in-human research: challenges for informed consent. *J. Law Med. Ethics* 40, 823–830. doi: 10.1111/j.1748-720X.2012.00710.x

Kola, I., and Landis, J. (2004). Can the pharmaceutical industry reduce attrition rates? *Nat. Rev. Drug Discov.* 3, 711–715. doi: 10.1038/nrd1470

Lammers, T., Aime, S., Hennink, W. E., Storm, G., and Kiessling, F. (2011). Theranostic nano medicine. *Acc. Chem. Res.* 44, 1029–1038. doi: 10.1021/ar200019c

Leist, M., and Hartung, T. (2013). Inflammatory findings on species extrapolations: humans are definitely No 70-kg mice. *Arch. Toxicol.* 87, 563–567. doi: 10.1007/s00204-013-1038-0

Nel, A. E. (2013). Implementation of alternative test strategies for the safety assessment of engineered nanomaterials. *J. Intern. Med.* 274, 561–577. doi: 10.1111/joim.12109

Nel, A. E., Nasser, E., Godwin, H., Avery, D., Bahadori, T., Bergeson, L., et al. (2013a). A multi-stakeholder perspective on the use of alternative test strategies for nanomaterial safety assessment. *ACS Nano* 7, 6422–6433. doi: 10.1021/nn4037927

Nel, A., Xia, T., Meng, H., Wang, X., Lin, S., Ji, Z., et al. (2013b). Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. *Acc. Chem. Res.* 46, 607–621. doi: 10.1021/ar300022h

Nelson, B. C., Wright, C. W., Ibuki, Y., Moreno-Villanueva, M., Karlsson, H. L., Hendriks, G., et al. (2017). Emerging metrology for high-throughput nanomaterial genotoxicology. *Mutagenesis* 32, 215–232. doi: 10.1093/mutage/gew037

Oberdorster, G. (2010). Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology. *J. Intern. Med.* 267, 89–105. doi: 10.1111/j.1365-2796.2009.02187.x

Oomen, A. G., Bos, P. M. J., Fernandes, T. F., Hund-Rinke, K., Boraschi, D., Byrne, H. J., et al. (2014). Concern driven integrated approaches to nanomaterial testing and assessment—report of the NanoSafety Cluster Working Group 10. *Nanotoxicology* 8, 334–348. doi: 10.3109/17435390.2013.802387

Oomen, A. G., Steinhäuserb, K. G., Bleekera, E. A. J., van Broekhuizena, F., Sipsa, A., Dekkersa, S., et al. (2018). Risk assessment frameworks for nanomaterials: scope, link to regulations, applicability, and outline for future directions in view of needed increase in efficiency. *NanoImpact* 9, 1–13. doi: 10.1016/j.impact.2017.09.001

Ramachandran, G., Howard, J., Maynard, A., and Philbert, M. (2012). Handling worker and third-party exposures to nanotherapeutics during clinical trials. *J. Law Med. Ethics* 40, 856–864. doi: 10.1111/j.1748-720X.2012.00714.x

Rasmussen, K., Rauscher, H., Mech, A., Riego Sintes, J., Gilliland, D., González, M., et al. (2018). Physico-chemical properties of manufactured nanomaterials - characterisation and relevant methods. An outlook based on the OECD Testing Programme. *Regul. Toxicol. Pharmacol.* 92, 8–28. doi: 10.1016/j.yrtph.2017.10.019

Resnik, D. B., and Tinkle, S. S. (2007). Ethics in nanomedicine. *Nanomedicine* 2, 345–350. doi: 10.2217/17435889.2.3.345

Satalkar, P., Elger, B. S., and Shaw, D. M. (2016). Defining nano, nanotechnology and nanomedicine: why should it matter? *Sci. Eng. Ethics* 22, 1255–1276. doi: 10.1007/s11948-015-9705-6

Schütz, C. A., Juillerat-Jeanneret, L., Mueller, H., Lynch, I., Riediker, M., and NanoImpactNet Consortium (2013). Therapeutic nanoparticles in clinics and under clinical evaluation. *Nanomedicine* 8, 449–467. doi: 10.2217/nnm.13.8

Senjen, R. (2013). *Nanomedicine: New Solutions or New Problems*? PhD, HCWH Europe; Mary Taylor.

Shatkin, J. A., and Ong, K. J. (2016). Alternative testing strategies for nanomaterials: state of the science and considerations for risk analysis. *Risk Anal.* 36, 1564–1580. doi: 10.1111/risa.12642.

Slikker, W., Andersen, M. E., Bogdanffy, M. S., Bus, J. S., Cohen, S. D., Conolly, R. B., et al. (2004). Dose-dependent transitions in mechanisms of toxicity. *Toxicol. Appl. Pharmacol.* 201, 203–225. doi: 10.1016/j.taap.2004.06.019

Stone, V., Pozzi-Mucelli, S., Tran, L., Aschberger, K., Sabella, S., Vogel, U., et al. (2014). ITS-NANO—Prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy. *Part. Fibre Toxicol.* 11:9. doi: 10.1186/1743-8977-11-9

Wagner, V., Dullaart, A., Bock, A.-K., and Zweck, A. (2006). The emerging nanomedicine landscape. *Nat. Biotechnol.* 24, 1211–1217. doi: 10.1038/nbt1006-1211

Winkler, D. A. (2016). Recent advances, and unresolved issues, in the application of computational modelling to the prediction of the biological effects of nanomaterials. *Toxicol. Appl. Pharmacol.* 299, 96–100. doi: 10.1016/j.taap.2015.12.016

Zhang, H., Ji, Z., Xia, T., Meng, H., Low-Kam, C., and Rong Liu, R. (2012). Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. *ACS Nano* 6, 4349–4368. doi: 10.1021/nn3010087

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REVIEW
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Current Trends and Challenges in the Clinical Translation of Nanoparticulate Nanomedicines: Pathways for Translational Development and Commercialization

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The use of nanotechnology in medicine has the potential to have a major impact on human health for the prevention, diagnosis, and treatment of diseases. One particular aspect of the nanomedicine field which has received a great deal of attention is the design and development of nanoparticulate nanomedicines (NNMs) for drug delivery (i.e., drug-containing nanoparticles). NNMs are intended to deliver drugs via various mechanisms: solubilization, passive targeting, active targeting, and triggered release. The NNM approach aims to increase therapeutic efficacy, decrease the therapeutically effective dose, and/or reduce the risk of systemic side effects. In order to move a NNM from the bench to the bedside, several experimental challenges need to be addressed. This review will discuss the current trends and challenges in the clinical translation of NNMs as well as the potential pathways for translational development and commercialization. Key issues related to the clinical development of NNMs will be covered, including biological challenges, large-scale manufacturing, biocompatibility and safety, intellectual property (IP), government regulations, and overall cost-effectiveness in comparison to current therapies. These factors can impose significant hurdles limiting the appearance of NNMs on the market, irrelevant of whether they are therapeutically beneficial or not.

Keywords: nanomedicine, nanoparticles, drug delivery systems, clinical translation, challenges, commercialization, biological, regulations

INTRODUCTION

Nanomedicine applies nanotechnology to highly specific medical interventions for the prevention, diagnosis, and treatment of diseases (Teli et al., 2010). In the last several decades, the application of nanotechnology for medical purposes has received significant attention from researchers, academia, funding agencies, government, and regulatory bodies (Allen and Cullis, 2004; Sercombe et al., 2015; Hare et al., 2017). One particular aspect of the nanomedicine field which has

received a great deal of attention is the design and development of nanoparticulate nanomedicines (NNMs) for drug delivery (i.e., drug-containing nanoparticles), which are most often given by parenteral (particularly intravenous) administration. NNMs are intended to increase the therapeutic index of drugs (i.e., increase efficacy and/or reduce toxicity) by delivering them via various mechanisms: solubilization, passive targeting, active targeting, and triggered release (**Figure 1**). Nanoencapsulation gives the opportunity to protect fragile compounds that degrade easily in biological environments and to provide solubilization, i.e., to deliver compounds which have physicochemical properties that strongly limit their aqueous solubility and therefore systemic bioavailability (Talekar et al., 2015; Kim et al., 2016; Larsson et al., 2017; Mishra et al., 2017; Shajari et al., 2017). Targeted drug delivery and triggered release of NNMs have been shown to be beneficial for increasing the therapeutic index of compounds, by improving the *in vivo* fate of drug molecules such that more efficient delivery to the target site is achieved (to yield improved therapeutic effects) with less accumulation in many healthy body sites (to reduce toxicity). Also NNMs have been studied for their ability to stimulate target cell uptake and improve intracellular trafficking, processes sometimes required when they have localized in target tissues (Mastrobattista et al., 1999; Hua, 2013; Hua et al., 2015).

Although NNMs have demonstrated significant therapeutic advantages for a multitude of biomedical applications, their clinical translation has not progressed as rapidly as the plethora of positive preclinical results would have suggested (Luxenhofer et al., 2014). In order to move a NNM from the bench to the bedside, several experimental challenges need to be addressed. From a biological perspective, these include studies focused on understanding the *in vivo* fate and interactions of NNMs with the blood, tissue, cellular, and intracellular compartments in the host in healthy and diseased states (Nehoff et al., 2014; Sercombe et al., 2015; Hare et al., 2017). For NNMs to have clinical translation potential, the complexity in their design and development also needs to be minimized as much as possible to create systems that are able to be reproducibly prepared and characterized (Lammers, 2013; Barz et al., 2015). This review will address the current trends and challenges in the clinical translation of NNMs as well as the potential pathways for translational development and commercialization.

CURRENT TRENDS IN THE CLINICAL TRANSLATION OF NANOMEDICINES

NNMs are often studied to improve drug targeting to specific sites of disease (i.e., site-specific drug delivery) and/or attenuate localization in healthy non-target tissues (i.e., site-avoidance drug delivery; Rizzo et al., 2013). The vast majority of NNMs in preclinical and clinical development as well in clinical use are for targeting a wide variety of cancers and tumors (Hare et al., 2017). The application of NNM-based therapies for drug targeting to non-cancer conditions has increased in recent years. In particular, NNMs have been developed to address the clinical challenge of effectively managing inflammatory diseases by exploiting the underlying biology of these conditions (Milane and Amiji, 2017). Non-cancerous inflammatory diseases that have been explored with NNM therapy include rheumatoid arthritis, inflammatory bowel disease, asthma, multiple sclerosis, diabetes, and neurodegenerative diseases (Milane and Amiji, 2017).

Enhanced Permeability and Retention (EPR) Effect and Passive Accumulation of NNMs

The EPR effect refers to the preferential localization of NNMs in pathological tissues due to the enhanced permeability of the vasculature that supplies such tissues (e.g., tumors and inflammatory conditions). Deregulations in angiogenesis and/or the increased expression and activation of vascular permeability factors predominates at these sites, which can lead to fenestrations allowing passage of NNMs (Hashizume et al., 2000; Nehoff et al., 2014). In addition to the enhanced leakiness of tumorous and inflamed blood vessels, the EPR effect also relates to the observation that solid tumors tend to lack functional lymphatic drainage, which limits the removal of extravasated NNMs from the target site (Maeda et al., 2013; Danhier, 2016). These pathological properties allow NNMs to accumulate at pathological sites and is referred to as passive targeting. To achieve this, it is important that NNMs with drug cargo circulate long enough in the bloodstream (i.e., show prolonged circulation kinetics). This can be achieved by conjugating polyethylene glycol (PEG) to the surface of NNMs (**Figure 1**). Thus, the EPR effect is expected to increase the therapeutic efficacy of NNMs in comparison to small molecules, which often show inferior pharmacokinetic properties (Matsumura and Maeda, 1986; Hobbs et al., 1998; van der Meel et al., 2013). The EPR effect was first observed in 1986 (Matsumura and Maeda, 1986) and has since been exploited particularly for the development of NNMs for passive tumor targeting, leading to NNMs with adequate physicochemical properties and prolonged circulation half-life that accumulate in tumors over time (Maeda et al., 2013; Nakamura et al., 2015; Danhier, 2016). The EPR effect and thus extent of passive targeting is highly dependent on the tumor pathophysiology. Currently, it is recognized that EPR is a very heterogeneous phenomenon as it depends on the type of tumor and can vary significantly within the same tumor type (Lammers et al., 2012; Ojha et al., 2017). The degree of tumor vascularization and passive targeting of NNMs has been observed to be positively correlated (Theek et al., 2014). For example, Doxil® (pegylated liposomal doxorubicin) is the first FDA-approved NNM and has demonstrated superior efficacy in ovarian cancer and AIDS-related Kaposi's sarcoma compared to standard conventional therapies (Nichols and Bae, 2014). When doxorubicin is encapsulated within PEGylated liposomes, it delays and minimizes uptake and clearance by the reticuloendothelial system (RES), thereby prolonging circulation half-life. This allows the NNM to accumulate in the tumor tissue by exploiting the locally increased permeability of the tumor blood vessels, rather than in non-target healthy tissues which do not have such leaky vessels (Rahman et al., 2007). Furthermore, the use of pegylated liposomal doxorubicin avoids high plasma

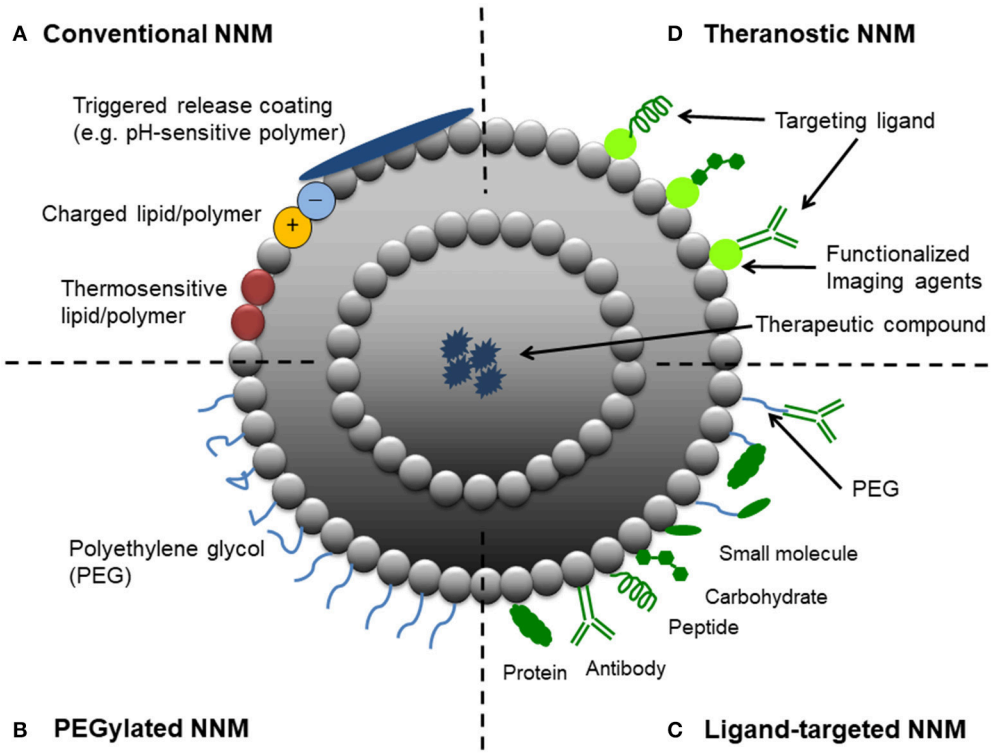

FIGURE 1 | Schematic representation of different strategic designs for nanoparticulate nanomedicines (NNMs). **(A)** Conventional NNM—These NNMs can be modified with charged lipids/polymers, thermosensitive lipids/polymers and/or components for triggered release (e.g., pH-sensitive coating). **(B)** PEGylated NNM—Nanoparticle characteristics and behavior *in vivo* can be modified by the addition of a hydrophilic polymer coating, polyethylene glycol (PEG), to the NNM surface to confer steric stabilization. **(C)** Ligand-targeted NNM—Nanoparticles can be used for active targeting by attaching ligands (e.g., antibodies, peptides and carbohydrates) to its surface or to the terminal end of the attached PEG chains. **(D)** Theranostic NNM – These NNM systems consist of an imaging component and a therapeutic component, and may include a targeting element.

peak levels of free drug (Lyass et al., 2000) and significantly reduces the risk of cardiotoxicity by preventing doxorubicin release through the heart vasculature (Rahman et al., 2007).

NNMs and Active Targeting

Active targeting, also termed ligand-targeting or receptor-mediated targeting, involves the use of ligands (e.g., antibodies, peptides or sugar moieties) which are physically or chemically conjugated onto the surface of NNMs to facilitate localization to and/or uptake by target cells (van der Meel et al., 2013; Danhier, 2016; **Figure 1**). Ligand-targeted NNMs have enormous potential for site-specific delivery of therapeutic compounds to designated cell types *in vivo,* which selectively express or over-express specific receptors (e.g., cellular receptors or cell adhesion molecules) at the site of disease (Willis and Forssen, 1998; Hua, 2013). For example, three sets of cellular targets are generally considered for active targeting in cancer—(i) targeting of cancer cells, which present overexpression of receptors for transferrin, folate, epidermal growth factor or glycoproteins; (ii) targeting of the tumor endothelium overexpressing vascular endothelial growth factors, integrins, vascular cell adhesion molecule-1 or matrix metalloproteinases; and (iii) targeting of stroma cells (e.g., macrophages, fibroblasts) that can acquire a tumor survival-promoting phenotype in response to cytokines in the tumor microenvironment (Coimbra et al., 2010; Danhier et al., 2010; Kuijpers et al., 2010; Danhier, 2016). There is still much debate about whether ligand-targeted NNMs are capable of significantly enhancing NNM accumulation at target sites over non-targeted NNMs (passive-targeting), with conflicting results reported in the literature (Ferrari, 2005; Puri et al., 2009; Riehemann et al., 2009; van der Meel et al., 2013). Enhanced therapeutic effects have been demonstrated with ligand-targeted NNMs, despite showing no differences in accumulation in target tissues compared to non-targeted NNMs. For example, similar high levels of tumor tissue accumulation were achieved with both non-targeted liposomes and liposomes conjugated with HER2 monoclonal antibody fragments (7–8% injected dose/g tumor tissue) in HER2-overexpressing breast cancer xenografts models (Kirpotin et al., 1997, 2006). However, significantly superior therapeutic results was demonstrated with the doxorubicin-loaded anti-HER2 immunoliposomes in comparison to all other control groups, including recombinant anti-HER2 Mab trastuzumab, non-targeted liposomal doxorubicin, and free doxorubicin (Park et al., 2002). Differences in pharmacodynamics of the targeted NNM formulation *in vivo* was suggested as the reason for the

improved anti-tumor effect, by enhancing intracellular drug delivery to HER2-overexpressing cancer cells (Kirpotin et al., 2006).

NNMs for Triggered Release

A third targeting strategy based on stimuli-responsive NNMs, referred to as triggered drug release, is currently receiving much attention from academia and industry. This class of NNMs is designed with the goal of enhancing drug release in tumors by means of endogenous or exogenous stimuli. Endogenous stimuli-responsive NNMs exploit factors associated with the local environment at the site of disease (**Figure 1**). For example, low pH, presence of redox gradients or certain enzymes in the tumor microenvironment. Exogenous-responsive NNMs respond to external stimuli to trigger drug release, such as temperature, light, magnetic field or ultrasound. Of these strategies, the use of an external hyperthermic trigger to release therapeutic compounds from NNMs (e.g., thermosensitive liposomal doxorubicin, ThermoDox®) appears to be the most promising to date (Needham et al., 2000). ThermoDox® was shown to be superior to its counterpart Doxil® in an *in vivo* model of non-resectable hepatocellular carcinoma (Torchilin, 2006; Sawant and Torchilin, 2012; Oude Blenke et al., 2013; Bertrand et al., 2014; Min et al., 2015; Jang et al., 2016; Shi et al., 2017). Thermosensitive liposomes are typically modified with temperature-sensitive lipids (e.g., distearoyl phosphocholine, DSPC) and/or polymers [e.g., poly(N-isopropylacrylamide)]. This composition allows the NNM to remain stable and retain their contents at physiologic temperatures, and undergo a phase change that makes them more permeable upon heating, thereby triggering the release of the cargo (Kono, 2001). The advantages of these NNMs can be further extended with the incorporation of imaging moieties (**Figure 1**) to enable monitoring of biodistribution, target accumulation and efficacy.

NNMs Approved and in Clinical Trials

A number of NNM products are on the market with more in clinical development. The majority of NNMs in clinical development incorporate already approved drugs and are based on a variety of drug delivery platforms, including polymeric micelles, liposomes, dendrimers, and inorganic nanoparticles (Torchilin, 2006; Wagner et al., 2006; Sercombe et al., 2015). Despite the arsenal of nanoparticulate targeted systems currently under preclinical development or in clinical trials, it is indisputable that liposomes are dominant on the NNM market (**Table 1**) and were the first FDA-approved NNM (Caster et al., 2017; Shi et al., 2017). In fact, liposomes have all the necessary features to allow formulation of highly toxic and/or poorly soluble drugs, such as paclitaxel and amphotericin B (Min et al., 2015; Caster et al., 2017). Soon after their discovery in 1965 (Sessa and Weissmann, 1968; Deamer, 2010), liposomes were proposed as drug delivery vehicles for both small molecules as well as macromolecular drugs (Gregoriadis and Ryman, 1971; Gregoriadis et al., 1971). Years of research led to the development of the first FDA-approved NNM (Doxil®/Caelyx®) as well as additional therapeutics (Allen and Cullis, 2013). Expectedly, many more NNMs are progressing to clinical investigation every year (**Table 2**), and again liposomal formulations represent the biggest share of the NNMs under clinical evaluation. The most frequently observed clinical benefit so far has been a reduction in toxicity with little evidence of improved efficacy. However, recently approved liposomal NNM, Vyxeos® (daunorubicin/cytarabine liposomal formulation), demonstrated improved survival and response rates, with tolerable toxicity in phase III clinical trials in older patients with therapy-related acute myeloid leukemia (t-AML) or AML with myelodysplasia-related changes (AML-MRC; Kim and Williams, 2018).

CHALLENGES IN THE CLINICAL TRANSLATION OF NANOMEDICINES

The clinical translation of NNMs is an expensive and time-consuming process. NNM technology is usually far more complex in comparison to conventional formulation technology containing free drug dispersed in a base (e.g., tablets, capsules and injections; Teli et al., 2010; Tinkle et al., 2014; Sainz et al., 2015). Key issues related to the clinical development of NNMs are listed in **Table 3**, and include biological challenges, large-scale manufacturing, biocompatibility and safety, intellectual property (IP), government regulations, and overall cost-effectiveness in comparison to current therapies (Allen and Cullis, 2004, 2013; Zhang et al., 2008; Sawant and Torchilin, 2012; Narang et al., 2013). These factors can impose significant hurdles limiting the appearance of NNMs on the market, irrelevant of whether they are therapeutically efficacious or not.

Biological Challenges

Traditionally, NNM development has been based on a formulation-driven approach, whereby novel delivery systems are firstly engineered and characterized from a physicochemical perspective. It is only when attempting to align the NNM with a pathological application that limitations in the clinical translation of the system have been identified. Understanding the relationship between biology and technology, including understanding the influence of disease pathophysiology on nanomedicine accumulation, distribution, retention and efficacy, as well as the biopharmaceutical correlation between delivery system properties and *in vivo* behavior in animals versus humans are important determinants for the successful translation of NNMs. Therefore, applying a disease-driven approach by designing and developing NNMs that are able to exploit pathophysiological changes in disease biology has been suggested to improve clinical translation (Hare et al., 2017).

From the outset in NNM development, it is essential to consider the relationship between disease pathophysiology and the heterogeneity of the disease in humans, and the importance of physicochemical characteristics of different NNMs to overcoming biological barriers to enable improved targeting to diseased tissue and/or reduced accumulation in non-target organs. Considerably less research effort has been dedicated to comprehensively understanding the correlations between NNM behavior and patient biology in specific clinical applications as well as disease heterogeneity in patients—which are likely

TABLE 1 | NNM formulations currently approved for marketing.

Type	Name	Drug	Indication
Liposomal NNMs	Doxil/Caelyx	Doxorubicin	HIV-related Kaposi's Sarcoma, metastatic breast cancer, advanced ovarian cancer, multiple myeloma
	AmBisome	Amphotericin B	Fungal infections
	DaunoXome	Daunorubicin	HIV-related Kaposi's Sarcoma
	Myocet	Doxorubicin	Metastatic breast cancer
	Abelcet	Amphotericin B	Fungal infections
	Lipo-Dox	Doxorubicin	HIV-related Kaposi's Sarcoma, ovarian cancer, multiple myeloma
	Marqibo (Onco-TCS)	Vincristine	Adult AML
	Onivyde	Irinotecan	Pancreatic cancer
	Vyxeos (CPX-351)	Cytarabine and daunorubicin	AML
	Visudyne	Verteporfin	Wet AMD, myopia, ocular histoplasmosis
	DepoDur	Morphine	Postoperative analgesia
	DepoCyt	Cytarabine	Lymphomatous meningitis
Micellar NNMs	Genexol PM	Paclitaxel	Metastatic breast cancer, advanced lung cancer
	Nanoxel M	Paclitaxel	Advanced NSCLC, breast cancer, pancreatic cancer, ovarian cancer
Protein NNMs	Abraxane	Paclitaxel	Breast cancer, NSCLC, pancreatic cancer

(Ref: ema.europa.eu; drugs.com; fda.gov).

the major reasons for the failure seen in the translation of promising NNMs in clinical trials (Hare et al., 2017). These biological challenges can be a significant deterrent for pharmaceutical industry investment into nanomedicines. In order to reduce investment risk for NNMs, the preclinical data sets need to comprehensively evaluate therapeutic efficacy, safety, biodistribution, and pharmacokinetics in appropriate animal models of the disease that are relevant to human disease. Evaluation of NNMs in multiple preclinical animal models that represent aspects of the clinical disease is preferred to achieve reproducibility of results for the specific disease and not for a specific animal model. In addition, animal models that reflect only a narrow spectrum of the clinical disease may provide useful data that can predict their suitability for treating a specific patient sub-group (Hare et al., 2017). Differences in the anatomy and/or physiology of the animal species compared to humans should be taken into account based on different routes of administration. Preclinical studies of NNMs should also be conducted under appropriate randomization and blinding to reduce bias, as well be evaluated against proper controls, including the gold standard treatment and not just free drug solution. These factors are currently lacking in many published studies, which makes it difficult to assess clinical applicability and translatability. Other considerations include designing preclinical studies to optimize NNM performance *in vivo*, dosing schedules, and treatment combinations based on the specific clinical disease, as well as understanding the influence of disease progression and severity on nanomedicine performance. This will determine whether specific patient sub-groups may respond more favorably to NNM-based treatment.

Interestingly, the majority of the NNM formulations in development and clinical trials are focused on cancer targeting, including more than 80% of the publications on nanomedicine in the last two decades alone (Park, 2017). Despite the large number of publications, the translation of the published studies to clinical applications has been disappointing. Cancer targeting of NNMs has generally been universally based on the EPR effect, despite the fact that EPR-mediated accumulation has only been reported for some tumor types (Maeda, 2015). Tumors, like other clinical diseases, can be highly heterogeneous and can show inter-patient and intra-patient variability as the disease progresses. Hence a one-size-fits-all approach when designing NNM-based treatment is unlikely to translate to clinically beneficial outcomes. The EPR effect has increasingly been exploited for NNM targeting in other non-cancer conditions, especially those involving an inflammatory component that causes leakiness of inflamed blood vessels (e.g., rheumatoid arthritis, atherosclerosis, and inflammatory bowel disease; Metselaar et al., 2003, 2004; Maiseyeu et al., 2009; Crielaard et al., 2012; Hua and Cabot, 2013; Hua et al., 2015; Milane and Amiji, 2017). It should be appreciated that not all diseases can be accessed with NNMs due to biological barriers and that the EPR effect is unlikely to be present in all clinical diseases. EPR is also not the only determinant of NNM efficacy. NNM activity is also influenced by the extent of cellular uptake and kinetics of drug release within target tissues (Hare et al., 2017).

Furthermore, the advantages of ligand-targeted NNMs in the clinical research phase have so far been negligible, despite the enhanced accumulation in target sites and therapeutic outcomes in a number of preclinical studies (Sercombe et al., 2015). Potential reasons for this discrepancy have previously been reviewed (Sawant and Torchilin, 2012; Allen and Cullis, 2013), and include factors such as target accessibility and expression, disease-dependent anatomical and physiological barriers, and formulation stability. In addition, the optimal targeting ligand density on the surface of each NNM has yet

TABLE 2 | NNM formulations in clinical trials.

Type	Name	Drug	Indication	Status
Lipid NNMs	LiPlaCis	Cisplatin	Advanced or refractory solid tumors, metastatic breast cancer and skin cancer	Phase I/II
	ThermoDox	Doxorubicin	Hepatocellular carcinoma, breast cancer	Phase I/IIII
	9NC-LP	9-Nitro-20 (S)-Camptothecin	Ewing's sarcoma and other solid tumors with lung involvement, endometrial cancer	Phase I/II completed
	SPI-077	Cisplatin	Ovarian cancer, relapsed/progressive osteosarcoma metastatic to the lung	Phase I/ II/ III
	Lipoxal	Oxaliplatin	Colorectal cancer, glioma	Phase II
	EndoTAG-1	Paclitaxel	Pancreatic cancer, liver metastases, HER2 and triple negative breast cancer	Phase II completed
	OSI-211	Lurtotecan	SCLC	Phase I/II completed
	LE-DT	Docetaxel	Solid tumors, pancreatic cancer	Phase I/II completed
	LEP-ETU	Paclitaxel	Breast cancer, neoplasm, gastric carcinoma	Phase I/II/IV
	TKM-080301	siRNA against PLK1	Advanced hepatocellular carcinoma, solid tumors or lymphomas that are refractory to conventional therapies; colorectal, gastric, breast and ovarian cancers with hepatic metastases	Phase I/II completed
	Atu027	siRNA against PKN3	Advanced solid tumors, pancreatic cancer	Phase I/II completed
	2B3-101	Doxorubicin	Advanced solid tumors, brain metastases, lung and breast cancers, melanoma, malignant glioma	Phase I/II completed
	MTL-CEBPA	saRNA	Liver cancer	Phase I
	TLI	Topotecan	SCLC, ovarian cancer, solid tumors	Phase I
	MM-398 Onivyde	Irinotecan	Solid tumors, ER/PR positive and triple negative breast cancer, metastatic breast cancer with active brain metastasis, SCLC, metastatic pancreatic cancer	Phase I/II/III
	MM-302	Doxorubicin	Breast cancer	Phase I
	ATI-1123	Docetaxel	Advanced solid tumors	Phase I completed
	SGT-53	p53 pDNA	Solid tumors, recurrent glioblastoma	Phase I/II
	SGT-94	RB94 pDNA	Solid tumors, recurrent glioblastoma	Phase I, Phase II
	Anti-EGFR-IL-DOX	Doxorubicin	Solid tumors	Phase II
	RNL	Rhenium-186	Glioblastoma and astrocytoma (treatment and imaging)	Phase I/II
	Patisiran	siRNA	TTR-mediated amyloidosis	Phase I/II/III
Polymeric NNMs	Paclical	Paclitaxel	Ovarian cancer	Phase III completed
	NK105	Paclitaxel	Gastric cancer	Phase III completed
	BIND-014	Docetaxel	NSCLC, solid tumors	Phase II completed
	CALAA-01	RRM2 siRNA	Solid tumors	Phase II terminated
	CRLX101	Camptothecin	NSCLC	Phase II completed

(Ref: clinicaltrials.gov).

to be determined and will likely depend on characteristics of the molecular target (e.g., expression, location, internalization rate and immunogenicity; Puri et al., 2009; Hua and Wu, 2013; Kraft et al., 2014). Detailed analysis of the degree of NNM accumulation, cellular internalization, intracellular functionality and intracellular degradation will also be important considerations for clinical validation and translation (Puri et al., 2009). Through extensive experimentation, we are gaining a better understanding of the more appropriate clinical applications for ligand-targeted NNMs. Therefore, by taking a disease-driven approach to NNM development, it will be possible to build comprehensive preclinical data sets that best predict efficacy for patient sub-groups and support translatable clinical development.

Large-Scale Manufacturing

One of the important factors contributing to the slow pace in the clinical translation of NNMs is the structural and physicochemical complexity of the formulation itself. Platforms that require complex and/or laborious synthesis procedures generally have limited clinical translation potential, as they can be quite problematic to pharmaceutically manufacture on a large-scale (Teli et al., 2010; Tinkle et al., 2014; Barz et al., 2015; Sainz et al., 2015). Pharmaceutical manufacturing development is centered on quality and cost. Quality includes the manufacturing process and stability of the formulation, with NNM manufacturing being challenged by potential issues related to: (i) poor quality control; (ii) scalability complexities; (iii) incomplete purification from contaminants (e.g., by-products

TABLE 3 | Considerations for the translational development of nanomedicines.

NANOPHARMACEUTICAL DESIGN
Key Considerations
▪ Route of administration
▪ Reduce complexity in formulation design
▪ Final dosage form for human use
▪ Biocompatibility and biodegradability
▪ Pharmaceutical stability (physical and chemical)
Current Obstacles
▪ Large-scale production according to GMP standards
○ *E.g., Reproducibility, infrastructure, techniques, expertise and cost*
▪ Quality control assays for characterization
○ *E.g., Size and polydispersity, morphology, charge, encapsulation, surface modifications, purity and stability*
PRECLINICAL EVALUATION
Key Considerations
▪ Need for validated and standardized assays for early detection of toxicity
▪ Evaluation in appropriate animal models of disease
▪ Adequate understanding of *in vivo* behavior, incl. cellular and molecular interactions
○ *Pharmacokinetics (absorption, distribution, metabolism and excretion)*
○ *Pharmacodynamics (intracellular trafficking, functionality, toxicity and degradation)*
Current Obstacles
▪ Development of more specialized toxicology studies for nanomedicines
▪ Adequate understanding of the interaction of NNM with tissues and cells
▪ Adequate structural stability of NNM following *in vivo* administration
▪ Limited degree of accumulation of nanomedicines in target organs/tissues/cells
CLINICAL EVALUATION FOR COMMERCIALIZATION
Key Considerations
▪ Simplification of development pathways from invention to commercialization to minimize time and expense
▪ Evaluation of safety/toxicity in humans (acute and chronic)
▪ Evaluation of therapeutic efficacy in patients
▪ Optimal clinical trial design
Current Obstacles
▪ Lack of clear regulatory guidelines specific for NNMs
▪ Complexity of NNM patents and IP
▪ Limited understanding of the biological interaction of NNM with the biological environment (incl. target site) in the body of patients

and starting materials); (iv) high material and/or manufacturing costs; (v) low production yield; (vi) insufficient batch-to-batch reproducibility, consistency and storage stability of the final product (e.g., regarding size distribution, porosity, charge and mass); (vii) lack of infrastructure and/or in-house expertise; (viii) chemical instability or denaturation of the encapsulated compound during the manufacturing process; and (ix) scarcity of venture funds and pharmaceutical industry investment (Teli et al., 2010; Narang et al., 2013; Hafner et al., 2014; Tinkle et al., 2014).

An essential requirement for clinical translation is to have access to a preparation method that allows the production of large scalable quantities of NNMs, which is also consistently manufactured at the same high level of quality and batch-to-batch reproducibility to set specifications (Grainger, 2013; Lammers, 2013; Barz et al., 2015). Suitable methods for the industrial scale production of several basic nanomedicine platforms, such as liposomes, have been successfully developed without the need for numerous manufacturing steps or the use of organic solvents (Jaafar-Maalej et al., 2012; Kraft et al., 2014). The challenges arise when the NNM system becomes more complex. For example, with the addition of surface modification with coatings and/or ligands, inclusion of multiple targeting components, or by encapsulating more than one therapeutic agent. Integration of multiple components into a single nanosized carrier requires multiple steps in the production process, which inevitably poses problems for large-scale good manufacturing (cGMP) production, increases the cost of production, and makes the quality assurance and quality control (QA and QC) evaluation of such products more difficult (Teli et al., 2010; Svenson, 2012; Tinkle et al., 2014).

Characteristics of the manufactured NNM need to be well-defined and reproducibly generated to allow initiation of clinical translation. Chemistry, Manufacturing, and Controls (CMC) information is required for investigational new drugs (IND) at each phase of investigation to ensure proper identity, strength or potency, quality, and purity of the drug substance and drug product (FDA, 2003). The type of information submitted will depend on the phase of the investigation, the extent of the human study, the duration of the investigation, the nature and source of the drug substance, and the drug product dosage form (FDA, 2003). The characterization and validation of more complex NNMs can be particularly challenging due to the sheer number of parameters to address (e.g., size distribution, morphology, charge, purity, drug encapsulation efficiency, coating efficiency, and density of conjugated ligand/s; Teli et al., 2010). Batch-to-batch variation of NNMs can potentially lead to significant changes to their physicochemical properties (e.g., polarity and size), pharmacokinetic parameters (i.e., absorption, distribution, metabolism and excretion), and/or pharmacodynamic interactions (e.g., cellular interaction and activity; Teli et al., 2010; Tinkle et al., 2014; Barz et al., 2015). In addition, NNMs need to be stable after the manufacturing process, during long-term storage, and upon clinical administration (i.e., to avoid massive drug release or aggregation in the bloodstream en route to the site of action).

Biocompatibility and Safety

Detailed toxicology is essential for the clinical translation of NNMs to determine the overall safety for human use (Nystrom and Fadeel, 2012). Pharmaceutical regulatory authorities generally recommend that the sponsor carefully assess for any changes in the drug substance and drug product manufacturing process or drug product formulation at any phase of clinical development, in order to determine if the changes can directly or indirectly affect the safety of the product. CMC modifications throughout the IND process that can affect safety include: (i) changes in the synthetic pathway or reagents used to manufacture the drug substance, product or formulation; (ii) changes resulting in a different impurity profile; (iii) changes in the actual manufacturing method (e.g., chemical synthesis, fermentation, or derivation from a natural source); (iv) changes in the source material; (v) changes in the method of sterilization of the drug substance or drug product; (vi) changes in the

route of administration; (vii) changes in the composition and/or dosage form of the drug product; (viii) changes in the drug product manufacturing process that can affect product quality; and (ix) changes in the drug product container closure system that can affect product quality (e.g., dose delivery; FDA, 2003). If any changes are identified, stringent procedures are in place to ensure appropriate comparison testing of the drug substance and/or drug product produced from the previous manufacturing process with the changed manufacturing process to evaluate product equivalency, quality, and safety (FDA, 2003). When analytical data demonstrate that the materials manufactured before and after are not comparable, sponsors should perform additional qualification and/or bridging studies to support the safety and bioavailability of the material to be used in the proposed trials (FDA, 2003).

Knowledge of the activity and toxicities of the free drug, the behavior of different NNM delivery systems and their interaction with biological components, and the influence of drug release rate on target and off-target concentrations of bioavailable drug allow the ability to predict potential side effects or toxicities *in vivo* (Hare et al., 2017). In particular, the rational design of NNMs from the early phase of material selection, production method optimization, and product purification is of fundamental importance to increase their clinical translation potential (Accomasso et al., 2018). Although the safety of some common materials such as phospholipids and biodegradable polymers have been studied previously (Storm et al., 1993), increasing the complexity of NNMs, such as the use of different synthetic compositions, coatings and ligands, can have a significant effect on the biocompatibility, biodistribution and toxicology profile of nanomedicines following *in vivo* administration (Allen and Cullis, 2004, 2013; Zhang et al., 2008; Sawant and Torchilin, 2012; Narang et al., 2013; Tinkle et al., 2014). For example, complement activation-related pseudoallergy (CARPA) is an acute adverse immune reaction caused by many NNMs (Szebeni, 2005; Sercombe et al., 2015; Szebeni and Storm, 2015; Jackman et al., 2016). The complement system is part of the innate immune response and is involved in a range of inflammatory and immunological processes (Moghimi and Hunter, 2001). CARPA is an immediate, non-IgE-mediated hypersensitivity reaction that can cause symptoms, including anaphylaxis, facial swelling, facial flushing, chills, headache, and cardiopulmonary distress (Szebeni, 2005). This adverse reaction is generally managed by slowing the infusion rate or ceasing therapy, as well as the use of standard allergy medications (e.g., antihistamines, corticosteroids and epinephrine; Sercombe et al., 2015; Szebeni and Storm, 2015). The development of immunogenic reactions to NNM-based therapies may lead to altered pharmacokinetics, loss of efficacy, and the rise of potentially serious toxicities (e.g., anaphylaxis; Szebeni and Moghimi, 2009; Szebeni and Storm, 2015).

There is a regulatory need for validated, sensitive and standardizable assays incorporating *in vitro, ex vivo* and *in vivo* protocols to appropriately assess the nanotoxicology of NNMs during the early stages of clinical development (Dobrovolskaia and McNeil, 2013; Jackman et al., 2016; Accomasso et al., 2018). Comprehensive *in vitro* or *ex vivo* assays for nanosafety testing are essential to screen for potential hazards prior to preclinical evaluation in animal models (Gaspar, 2007). For example, standardized *in vitro* protocols using different cell culture models (i.e., blood, liver, lung, brain, placenta, gastrointestinal system) to assess potential risk of cytotoxicity, immunotoxicity, and genotoxicity of NNMs (Accomasso et al., 2018). This is particularly important with the development of NNMs incorporating many new materials with the goal for use in the clinical setting. In order to do this effectively across the board, standardized reference materials would need to be established and the testing would also need to be relevant for the intended route of administration (Tinkle et al., 2014). Although current testing approaches are limited and insufficient for nanotoxicology evaluations for clinical translation, a number of techniques that are more specific for nanomedicines are under development. This includes alternative test strategies, high-throughput screening techniques, high-content screening, and computational modeling (Nel et al., 2013; Oomen et al., 2014; Dusinska et al., 2015; Accomasso et al., 2018). These techniques have the potential to analyze in a comparative way many NNMs simultaneously.

There is also a need to perform specialized toxicology studies in animal models to assess both short-term and long-term toxicity, as circulation half-lives and drug retention times are generally significantly increased with nanoencapsulation. A thorough understanding of the absorption, distribution, metabolism, and excretion of emerging nanomaterials *in vivo* is important to predict the toxicological responses to NNMs (Dobrovolskaia and McNeil, 2013; Tinkle et al., 2014). Adequate assessment protocols are needed to monitor various aspects of the NNM drug delivery process, including pharmacokinetics, biodistribution, target site accumulation, local distribution at the target site, localization in healthy tissues, kinetics of drug release, and therapeutic efficacy (Kunjachan et al., 2015). Incorporation of real-time imaging techniques have enabled better understanding of the interaction of NNMs with biological organs and tissues following *in vivo* administration (Gaspar, 2007; Nystrom and Fadeel, 2012; Dobrovolskaia and McNeil, 2013; Kunjachan et al., 2015).

In addition, biocompatibility, immunotoxicological, and inflammatory potential should be assessed, with functional outcomes correlated with mechanisms of tissue uptake and clearance (Gaspar, 2007). These parameters need to be well-investigated based on dose, dosage form and route of administration to establish safe limits prior to clinical trials (Gaspar, 2007; Nystrom and Fadeel, 2012). This is of particular importance for NNMs composed of materials that have never been used before in clinical applications. Even in the clinical trial phase, regulatory protocols should be in place to detect any toxicity caused not only by the encapsulated therapeutic compounds, but also novel mechanisms unique to nanotechnology (Gaspar, 2007; Nystrom and Fadeel, 2012). For example, short- and long-term effects of NNM accumulation in RES organs (esp. liver, kidneys, spleen, lungs, lymph nodes, and bone marrow; Senior, 1987; Szebeni and Barenholz, 2009; Szebeni and Moghimi, 2009), which are the main sites for NNM accumulation following systemic administration (Poste et al.,

1976; Senior, 1987). The cells of the RES are also part of the innate immune system, which has raised concerns regarding whether macrophage saturation by NNMs can cause immunosuppression and increase the risk of infections (Sercombe et al., 2015; Liu et al., 2017). There have been no reports of clinically significant immunosuppression at therapeutic doses of non-cytotoxic NNMs, despite suggestions that excessive NNM deposition in macrophages may impair their phagocytic capacity or modulate other cellular functions (Szebeni and Barenholz, 2009; Szebeni and Moghimi, 2009). However, NNMs that contain cytotoxic compounds are capable of inducing macrophage destruction following uptake (Szebeni and Barenholz, 2009; Szebeni and Moghimi, 2009), with indirect signs that suggest the possibility of some immune suppression (Storm et al., 1998; Szebeni and Barenholz, 2009; Szebeni and Moghimi, 2009). For example, administration of Doxil® in mice was reported to interfere with the clearance of bacteria from the blood due to macrophage suppression (Storm et al., 1998; Szebeni and Barenholz, 2009). Addressing these issues are necessary to safeguard the application of emerging NNMs in the clinical setting.

Intellectual Property (IP)

Given the complexities of incorporating nanotechnology into biomedical and clinical applications, there needs to be more precise definitions on what constitutes novel IP of a nanomedicine (Satalkar et al., 2015). Nanomedicines are complex as they have a number of variable components, and bridge between the field of medicine and medical device (Paradise et al., 2009). Generally, the control of a NNM product requires an IP position on: (i) the encapsulated cargo; (ii) the carrier technology; and (iii) the characteristics of the drug and carrier together. Although this definition is straightforward, it does open up a number of problems with the issuing of patents to date (Bawa, 2007; Bawa et al., 2008). For example, NNMs that incorporate existing drugs with novel carrier technology, or those that incorporate existing drugs with existing carrier technology for a new biomedical or disease application. The IP situation becomes even more confusing with more complex drug delivery systems, such as those which incorporate commercially available targeting ligands (e.g., antibodies) or coatings (e.g., Eudragit®) that are owned by other companies. IP strategies may likely involve multiple patents associated with any given technology and the need for cross-licensing arrangements (Murday et al., 2009). Therefore, new IP practices and protocols are required to simplify the pathway from invention to commercialization to reduce the time and expense required for negotiating collaboration and licensing agreements (Murday et al., 2009).

With the significant increase in the number of nanotechnology patent applications over the last few decades, other key issues that need to be addressed include patent review delays, patent thickets, and issuance of invalid patents (Bawa, 2005, 2007; Bawa et al., 2005). There needs to be a universal nano-nomenclature on identical or similar nanostructures or nanomaterials, and more refined search tools and commercial databases to avoid the issuing of multiple nanopatents on the same invention (Bawa et al., 2005; Bawa, 2007). Databases used by the Patent and Trademark Office (PTO) need to be able to search through nanotech-related prior art that resided in scientific publications world-wide, including earlier publications that preceded the emergence of online publication databases (Tinkle et al., 2014). Patent examiners also require expertise and training with respect to the emerging fields of nanotechnology and nanomedicine. The complexities with nanotechnology have led to the so called "patent thickets", which can lead to costly litigation and halt commercialization efforts (Tinkle et al., 2014). Therefore, improved clarity on IP and patenting surrounding nanotechnology in health and medicine is required, and will need to involve implementation of universal regulations and policies that are tailored toward this niche commercialization field.

Government Regulations

Nanomedicines have significant potential to increase the growth of the pharmaceutical market and improve health benefits, however the current scientific and regulatory gap for nanomedicines is large and challenging. Commercialization of nanomedicines is highly dependent on a number of regulatory factors based on government policies in the area of manufacturing practice, quality control, safety, and patent protection (Gaspar, 2007; Tinkle et al., 2014; Sainz et al., 2015). The lack of clear regulatory and safety guidelines has affected the development of NNM products toward timely and effective clinical translation (Gaspar, 2007; Tinkle et al., 2014; Sainz et al., 2015). For example, polymers have been widely investigated as an effective platform for NNM strategies; however, their safety and efficacy is highly dependent on the polymer molecular weight, polydispersity, molecular structure, and conjugation chemistry (Gaspar and Duncan, 2009; Diab et al., 2012). Due to the increased number of novel polymeric materials and complex polymeric-based NNM formulations, there is an urgent need for an appropriate regulatory framework to assist in evaluation (Gaspar and Duncan, 2009). As each polymer-based NNM is different, it is important to consider each individually based on doses, administration routes, dosing frequency, and proposed clinical use. This would be the same for most other NNM platforms.

NNMs are currently regulated within the conventional framework governed by the key regulatory authority of each country (e.g., FDA, TGA, and EMA). Although NNMs have been on the market for nearly two decades, the first generation of NNM products passed regulatory approval by only having to meet general standards, applicable to medicinal compounds. These regulations are no longer appropriate to confirm the quality, safety, and efficacy of NNMs for clinical use (Gaspar, 2007; Tinkle et al., 2014; Sainz et al., 2015). Reasons for this are based on the complex structure of NNMs, their unclear interaction with cells and tissues within the human body, increased complexity of clinical use, and the multifunctional nature of some formulations (e.g., integration of therapeutics with imaging diagnostics; Gaspar, 2007; Tinkle et al., 2014; Sainz et al., 2015). Regulatory standards and protocols validated specifically for nanoparticles are needed that bridge both medicine and medical devices regulations. This should take into account a NNM's complexity, route of administration, pharmacokinetics, pharmacodynamics and safety profile, as well

as provide information on the most appropriate clinical trial design and patient selection (Tinkle et al., 2014). There needs to be a fine balance to ensure the safety and quality of NNMs without over-regulation, which can negatively affect the progress of innovative products to the market, by escalating costs for achieving regulatory approval and/or consuming a significant portion of the life of a patent.

Development of global regulatory standards for NNMs should be established alongside key countries with invested interest. Although major steps have been taken in the last 5 years, a closer collaboration between regulatory agencies, academia, research and industry is needed (Gaspar, 2007; Murday et al., 2009; Hafner et al., 2014). This is of particular importance due to the limited availability of contract manufacturing organizations world-wide that specialize in producing NNM products in accordance with the requirements for good manufacturing practice (GMP; Hafner et al., 2014). It should be noted that this limited number of manufacturing organizations may be further divided based on their infrastructure capabilities of producing specific NNM platforms (e.g., liposomes, polymeric nanoparticles, dendrimers and drug-polymer conjugates). Therefore, NNMs produced in these manufacturing organizations will likely be marketed in multiple countries and thus should be governed under the same regulatory standards (Hafner et al., 2014). There will need to be complete evaluation and documentation of production processes for NNMs, incorporating appropriate industrial standards for both quality control and prevention of environmental issues (Gaspar, 2007). Manufactured NNMs will still need to meet general pharmaceutical standards such as purity, sterility, stability, manufacturing operations, and related industrial control standards (Gaspar, 2007). In addition, new analytical tools and standardized methods will need to be implemented to evaluate key physical characteristics of NNMs that can affect *in vivo* performance such as particle size and size distribution, surface chemistry, morphology, surface area, surface coating, hydrophilicity, porosity, and surface charge density (Gaspar, 2007; Tinkle et al., 2014; Sainz et al., 2015). These methods will vary for different nanomaterials and nanostructures. Thus, regulatory authorities should work together to develop the testing methods and appropriate standardized protocols for toxicity studies and regulatory requirements, which will be needed to ensure the efficacy and safety of current and emerging NNMs.

PERSPECTIVES ON THE TRANSLATIONAL DEVELOPMENT OF NANOMEDICINES

From a therapeutic perspective, increasing drug accumulation at target tissues and minimizing systemic adverse effects are still the biggest design challenges to meet when developing new drug delivery systems. Even though promising NNMs may demonstrate significant efficacy in *in vitro* or *ex vivo* studies, it is important to evaluate the platforms *in vivo* using appropriate animal models of the disease. It is here where many of the current NNM platforms have shown limited specificity, accumulation and/or stability, therefore providing unsatisfactory results to warrant progression in the R&D process (Hua et al., 2015). Efficacy in an animal model also does not necessarily equate to efficacy in humans, as drug delivery within the human body is complex and can be highly variable, especially when associated with disease (Hare et al., 2017). Therefore, this concept of designing nanomedicines that act like a "magic bullet," which refers to the exclusive delivery of active compounds to specific organs, tissues or cells, is just not realistic when taking into account the pharmacokinetic and pharmacodynamic processes that occur following administration into the body (Barz et al., 2015). This term should refer to the development of realistic therapeutic platforms, in which therapeutic effects are maximized, doses are minimized, and complexity in dosage form design is reduced (Barz et al., 2015).

Complexity in dosage form design is a key factor in the ability for a NNM formulation to be translated to the clinic, irrelevant of its therapeutic efficacy. Simplification in formulation design is required to allow efficient and reproducible large-scale manufacturing (Grainger, 2013; Lammers, 2013; Barz et al., 2015). Any added complexities to the basic NNM platform would need to show significantly improved benefits that is reliable and reproducible in animal models and patients, due to the added costs and complexity in the manufacturing process. For example, further studies are required to examine the benefits of ligand-targeted delivery systems over basic NNM platforms, in particular the reliability and consistency of the expression of the target across disease severity and in different patients (Hua et al., 2015; Sercombe et al., 2015; Hare et al., 2017). In addition, when translating findings from animal models to humans, we need to determine how to modify these formulations so that they are appropriate for human administration (Hua et al., 2015). *In vivo* studies are typically conducted in animal models of experimental diseases, especially in mice and rats, which can place limitations on the size and consistency of the dosage form that can be administered—for example, via oral, topical or intraperitoneal delivery (Hua et al., 2015; Sercombe et al., 2015). The practicability of designing dosage forms that are both acceptable to humans and efficacious should be further explored for clinical studies. Thus, there needs to be a balance between complexity, therapeutic efficacy, and clinical translation.

To transition NNMs to the clinic, attention should be given to nanosized carriers that are stable following *in vivo* administration, easily able to be up-scaled for manufacturing with high control over their physicochemical properties (e.g., size and polydispersity, morphology, drug encapsulation efficiency, and charge), as well as being composed of materials that are biocompatible, biodegradable, and non-toxic. As nanoparticles are able to enter cells and interfere with molecular pathways, synthetic polymers and lipids should be carefully evaluated for potential short-term and long-term toxicity for clinical application (Gaspar and Duncan, 2009). For example, potentially toxic *in vitro* and *in vivo* effects have been identified with the use of cationic polymers and lipids, including reduced number of mitoses, cell shrinking, detrimental effects on key cellular proteins (e.g., protein kinase C), and vacuolization of the cytoplasm (Lv et al., 2006).

PATHWAY TO TRANSLATION AND COMMERCIALIZATION

The experimental development of NNMs is progressing at a fast pace, however significant challenges still exist in promoting these platforms into clinically feasible therapies (**Table 3**). The majority of NNMs in the clinic are for the treatment of cancer, predominantly by the parenteral route of administration. They are structurally based on simple nanomedicine platforms, in particular basic nanoparticles, surface charge-modified nanoparticles, and PEGylated nanoparticles (Hafner et al., 2014; Sainz et al., 2015). Although clinical applications of nanotechnology for non-cancer diseases are increasing based on promising experimental results, there are several barriers that have slowed progress in the preclinical and, especially, clinical stages of development. This includes issues surrounding complexity in manufacturing and characterization, lack of understanding of *in vivo* pharmacokinetics and pharmacodynamics, acute and chronic toxicity, and cost-effectiveness (Gaspar, 2007; Teli et al., 2010; Hafner et al., 2014; Tinkle et al., 2014; Sainz et al., 2015). These challenges are even greater with increasing complexity of the NNM design.

The pace for the clinical translation of NNMs has been relatively slow as the development trajectory is very costly, complex and time-consuming, which has affected the attitudes of the pharmaceutical industry and capital investors. There has to be a clear positive benefit-to-risk ratio that will accompany the clinical implementation of products and procedures based on nanotechnology. In particular, the cost-benefit analysis may be a limitation to the clinical translation of some NNMs when compared to an approved counterpart or existing therapies. This analysis has to be clear before starting the development process. Emerging NNM products, which are more complex in structure and more expensive than conventional therapies, need to provide an overall reduction in health care costs and provide a worthwhile opportunity for the pharmaceutical industry to invest its R&D budgets (Hafner et al., 2014). This reduction in health care costs is likely to be obtained by increasing therapeutic efficacy, improving quality of life, reducing adverse effects or toxicities in non-target organs, and/or reducing the need for surgical or other high-risk interventions (Gandjour and Chernyak, 2011). Nanopharmaceuticals can offer the ability to extend the economic life of proprietary drugs and create additional revenue streams (Tinkle et al., 2014). In addition, market analysis, investment risk, potential profit margins, and value proposition of novel NNMs are important factors for the pharmaceutical industry and investors. Typically, pharmaceutical products that are developed to address larger disease populations with treatment expected in a primary or secondary care setting are preferred by the pharmaceutical industry. From a business perspective, the necessary infrastructure, understanding of NNMs, and skill set required for the commercial development of NNMs are not currently well represented at most pharmaceutical companies. These factors should be taken into account when assessing the overall cost-effectiveness of NNMs in comparison to existing therapies.

Nanomedicines generally face a number of regulatory approval hurdles. The control of materials in the nanosize range often presents greater scientific and technical challenges compared to conventional formulations (Gaspar, 2007; Teli et al., 2010; Hafner et al., 2014; Tinkle et al., 2014; Sainz et al., 2015). NNMs encompass a number of different types of nanomaterials and nanostructures, which make it even more challenging to establish appropriate regulatory protocols and tools to ensure standardized GMP manufacturing and characterization, safety and toxicology evaluation, and clinical trial design. These procedures are paramount to confirming therapeutic efficacy and safety prior to marketing approval for use in patients on a larger scale. Effective clinical translation will require an interdisciplinary approach to develop novel protocols, assays and infrastructure for the manufacturing and characterization of NNMs (Gaspar, 2007; Teli et al., 2010; Hafner et al., 2014; Tinkle et al., 2014; Sainz et al., 2015). This will need to involve experts from academia and industry with specialty in pharmaceutics, engineering, biology, medicine, and toxicology. Potential approaches to fast-track promising novel NNMs to clinical trials include the establishment or coordination of laboratories and centers that have expertise in (i) characterizing NNM platforms, (ii) conducting preclinical studies on NNMs for submission to regulatory agencies, (iii) scale up laboratory preparation of nanomaterials according to regulatory and industry standards for early clinical trials, and (iv) designing and conducting clinical trials of NNM platforms (Hafner et al., 2014).

CONCLUSION

Overall, the use of nanotechnology in medicine has the potential to have a major impact on human health. It has been suggested to facilitate the development of personalized medicine for specific patient sub-groups, in which therapy is tailored by the patient's individual genetic and disease profile (Teli et al., 2010; Mura and Couvreur, 2012; Laroui et al., 2013). For example, disease-specific characteristics such as capillary permeability (Calcagno et al., 2015), cellular receptor expression and molecular pathway activation could be analyzed and used to design personalized nanomedicines (Teli et al., 2010; Mura and Couvreur, 2012; Laroui et al., 2013). The physicochemical properties (e.g., size and structure) of the delivery system can also be modified according to the severity of the disease for optimal therapeutic benefits (Hua et al., 2015). This concept would significantly advance the way in which we treat patients. However, for this to occur, there are still a number of issues that need to be addressed as detailed in this review—from our basic understanding of the biology of specific diseases and the biological interaction of NNMs in patients, to commercialization hurdles related to manufacturing, costs, and regulatory standards. Finally, researchers need to consider minimizing the complexity of NNMs and take into account the final dosage form for human use, in order for a formulation to have the potential to be translated into a clinically applicable therapeutic. Reducing complexity to the minimum required for pathophysiological or medical need is paramount in nanoparticle

design and synthesis to generate clinically translatable nanosized therapeutics.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Accomasso, L., Cristallini, C., and Giachino, C. (2018). Risk Assessment and Risk Minimization in Nanomedicine: A Need for Predictive, Alternative, and 3Rs Strategies. *Frontiers in Pharmacology*. 9:228. doi: 10.3389/fphar.2018.00228

Allen, T. M., and Cullis, P. R. (2004). Drug delivery systems: entering the mainstream. *Science* 303, 1818–1822. doi: 10.1126/science.1095833

Allen, T. M., and Cullis, P. R. (2013). Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Del. Rev*. 65, 36–48. doi: 10.1016/j.addr.2012.09.037

Barz, M., Luxenhofer, R., and Schillmeier, M. (2015). Quo vadis nanomedicine? *Nanomedicine* 10, 3089–3091. doi: 10.2217/nnm.15.156

Bawa, R. (2005). Will the nanomedicine "patent land grab" thwart commercialization? *Nanomedicine* 1, 346–350. doi: 10.1016/j.nano.2005.10.009

Bawa, R. (2007). Patents and nanomedicine. *Nanomedicine* 2, 351–374. doi: 10.2217/17435889.2.3.351

Bawa, R., Bawa, S. R., Maebius, S. B., Flynn, T., and Wei, C. (2005). Protecting new ideas and inventions in nanomedicine with patents. *Nanomedicine* 1, 150–158. doi: 10.1016/j.nano.2005.03.009

Bawa, R., Melethil, S., Simmons, W. J., and Harris, D. (2008). Nanopharmaceuticals - patenting issues and FDA regulatory challenges. *Am. Bar Assoc. SciTech Lawyer*. 5, 10–15.

Bertrand, N., Wu, J., Xu, X., Kamaly, N., and Farokhzad, O. C. (2014). Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology. *Adv. Drug Deliv. Rev*. 66, 2–25. doi: 10.1016/j.addr.2013.11.009

Calcagno, C., Lobatto, M. E., Dyvorne, H., Robson, P. M., Millon, A., Senders, M. L., et al. (2015). Three-dimensional dynamic contrast-enhanced MRI for the accurate, extensive quantification of microvascular permeability in atherosclerotic plaques. *NMR Biomed*. 28, 1304–1314. doi: 10.1002/nbm.3369

Caster, J. M., Patel, A. N., Zhang, T., and Wang, A. (2017). Investigational nanomedicines in 2016: a review of nanotherapeutics currently undergoing clinical trials. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol*. 9:e1416. doi: 10.1002/wnan.1416

Coimbra, M., Banciu, M., Fens, M. H., de Smet, L., Cabaj, M., Metselaar, J. M., et al. (2010). Liposomal pravastatin inhibits tumor growth by targeting cancer-related inflammation. *J. Control Release*. 148, 303–310. doi: 10.1016/j.jconrel.2010.09.011

Crielaard, B. J., Lammers, T., Schiffelers, R. M., and Storm, G. (2012). Drug targeting systems for inflammatory disease: one for all, all for one. *J. Control Release*. 161, 225–234. doi: 10.1016/j.jconrel.2011.12.014

Danhier, F. (2016). To exploit the tumor microenvironment: since the EPR effect fails in the clinic, what is the future of nanomedicine? *J. Control. Release*. 244 (Pt A), 108–121. doi: 10.1016/j.jconrel.2016.11.015

Danhier, F., Feron, O., and Preat, V. (2010). To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. *J. Control Release*. 148, 135–146. doi: 10.1016/j.jconrel.2010.08.027

Deamer, D. W. (2010). From "banghasomes" to liposomes: a memoir of Alec Bangham, 1921-2010. *FASEB J*. 24, 1308–1310. doi: 10.1096/fj.10-0503

Diab, R., Jaafar-Maalej, C., Fessi, H., and Maincent, P. (2012). Engineered nanoparticulate drug delivery systems: the next frontier for oral administration? *AAPS J*. 14, 688–702. doi: 10.1208/s12248-012-9377-y

Dobrovolskaia, M. A., and McNeil, S. E. (2013). Understanding the correlation between *in vitro* and *in vivo* immunotoxicity tests for nanomedicines. *J Control Release*. 172, 456–466. doi: 10.1016/j.jconrel.2013.05.025

Dusinska, M., Boland, S., Saunders, M., Juillerat-Jeanneret, L., Tran, L., Pojana, G., et al. (2015). Towards an alternative testing strategy for nanomaterials used in nanomedicine: lessons from NanoTEST. *Nanotoxicology* 9 (Suppl. 1), 118–132. doi: 10.3109/17435390.2014.991431

FDA (2003).*Guidance for Industry - INDs for Phase 2 and Phase 3 Studies: Chemistry, Manufacturing, and Control (CMC) Information*

Ferrari, M. (2005). Nanovector therapeutics. *Curr. Opin. Chem. Biol*. 9, 343–346. doi: 10.1016/j.cbpa.2005.06.001

Gandjour, A., and Chernyak, N. (2011). A new prize system for drug innovation. *Health Policy*. 102, 170–177. doi: 10.1016/j.healthpol.2011.06.001

Gaspar, R. (2007). Regulatory issues surrounding nanomedicines: setting the scene for the next generation of nanopharmaceuticals. *Nanomedicine* 2, 143–147. doi: 10.2217/17435889.2.2.143

Gaspar, R., and Duncan, R. (2009). Polymeric carriers: preclinical safety and the regulatory implications for design and development of polymer therapeutics. *Adv. Drug Deliv. Rev*. 61, 1220–1231. doi: 10.1016/j.addr.2009.06.003

Grainger, D. W. (2013). Connecting drug delivery reality to smart materials design. *Int. J. Pharm*. 454, 521–524. doi: 10.1016/j.ijpharm.2013.04.061

Gregoriadis, G., Leathwood, P. D., and Ryman, B. E. (1971). Enzyme entrapment in liposomes. *FEBS Lett*. 14, 95–99. doi: 10.1016/0014-5793(71)80109-6

Gregoriadis, G., and Ryman, B. E. (1971). Liposomes as carriers of enzymes or drugs: a new approach to the treatment of storage diseases. *Biochem. J*. 124:58P. doi: 10.1042/bj1240058P

Hafner, A., Lovric, J., Lakos, G. P., and Pepic, I. (2014). Nanotherapeutics in the EU: an overview on current state and future directions. *Int. J. Nanomedicine*. 9, 1005–1023. doi: 10.2147/IJN.S55359

Hare, J. I., Lammers, T., Ashford, M. B., Puri, S., Storm, G., and Barry, S. T. (2017). Challenges and strategies in anti-cancer nanomedicine development: an industry perspective. *Adv. Drug. Deliv. Rev*. 108, 25–38. doi: 10.1016/j.addr.2016.04.025

Hashizume, H., Baluk, P., Morikawa, S., McLean, J. W., Thurston, G., Roberge, S., et al. (2000). Openings between defective endothelial cells explain tumor vessel leakiness. *Am. J. Pathol*. 156, 1363–1380. doi: 10.1016/S0002-9440(10)65006-7

Hobbs, S. K., Monsky, W. L., Yuan, F., Roberts, W. G., Griffith, L., Torchilin, V. P., et al. (1998). Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. *Proc. Natl. Acad. Sci. U.S.A*. 95, 4607–4612. doi: 10.1073/pnas.95.8.4607

Hua, S. (2013). Targeting sites of inflammation: intercellular adhesion molecule-1 as a target for novel inflammatory therapies. *Front. Pharmacol*. 4:127. doi: 10.3389/fphar.2013.00127

Hua, S., and Cabot, P. J. (2013). Targeted nanoparticles that mimic immune cells in pain control inducing analgesic and anti-inflammatory actions: a potential novel treatment of acute and chronic pain condition. *Pain Physician*. 16, E199–E216.

Hua, S., Marks, E., Schneider, J. J., and Keely, S. (2015). Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: Selective targeting to diseased versus healthy tissue. *Nanomedicine* 11, 1117–1132. doi: 10.1016/j.nano.2015.02.018

Hua, S., and Wu, S. Y. (2013). The use of lipid-based nanocarriers for targeted pain therapies. *Front. Pharmacol*. 4:143. doi: 10.3389/fphar.2013.00143

Jaafar-Maalej, C., Elaissari, A., and Fessi, H. (2012). Lipid-based carriers: manufacturing and applications for pulmonary route. *Expert Opin. Drug Deliv*. 9, 1111–1127. doi: 10.1517/17425247.2012.702751

Jackman, J. A., Meszaros, T., Fulop, T., Urbanics, R., Szebeni, J., and Cho, N. J. (2016). Comparison of complement activation-related pseudoallergy in miniature and domestic pigs: foundation of a validatable immune toxicity model. *Nanomedicine* 12, 933–943. doi: 10.1016/j.nano.2015.12.377

Jang, B., Kwon, H., Katila, P., Lee, S. J., and Lee, H. (2016). Dual delivery of biological therapeutics for multimodal and synergistic cancer therapies. *Adv. Drug Deliv. Rev*. 98, 113–133. doi: 10.1016/j.addr.2015.10.023

Kim, H. J., Kim, A., Miyata, K., and Kataoka, K. (2016). Recent progress in development of siRNA delivery vehicles for cancer therapy. *Adv. Drug. Deliv. Rev*. 104, 61–77. doi: 10.1016/j.addr.2016.06.011

Kim, M., and Williams, S. (2018). Daunorubicin and cytarabine liposome in newly diagnosed therapy-related Acute Myeloid Leukemia (AML) or AML with myelodysplasia-related changes. *Ann. Pharmacother*. 1:1060028018764923. doi: 10.1177/1060028018764923

Kirpotin, D. B., Drummond, D. C., Shao, Y., Shalaby, M. R., Hong, K., Nielsen, U. B., et al. (2006). Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. *Cancer Res*. 66, 6732–6740. doi: 10.1158/0008-5472.CAN-05-4199

Kirpotin, D., Park, J. W., Hong, K., Zalipsky, S., Li, W. L., Carter, P., et al. (1997). Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells *in vitro*. *Biochemistry* 36, 66–75. doi: 10.1021/bi962148u

Kono, K. (2001). Thermosensitive polymer-modified liposomes. *Adv. Drug Deliv. Rev*. 53, 307–319. doi: 10.1016/S0169-409X(01)00204-6

Kraft, J. C., Freeling, J. P., Wang, Z., and Ho, R. J. (2014). Emerging research and clinical development trends of liposome and lipid nanoparticle drug delivery systems. *J. Pharm. Sci*. 103, 29–52. doi: 10.1002/jps.23773

Kuijpers, S. A., Coimbra, M. J., Storm, G., and Schiffelers, R. M. (2010). Liposomes targeting tumour stromal cells. *Mol. Membr. Biol*. 27, 328–340. doi: 10.3109/09687688.2010.522204

Kunjachan, S., Ehling, J., Storm, G., Kiessling, F., and Lammers, T. (2015). Noninvasive imaging of nanomedicines and nanotheranostics: principles, progress, and prospects. *Chem. Rev*. 115, 10907–10937. doi: 10.1021/cr500314d

Lammers, T. (2013). Smart drug delivery systems: back to the future vs. clinical reality. *Int. J. Pharm*. 454, 527–529. doi: 10.1016/j.ijpharm.2013.02.046

Lammers, T., Kiessling, F., Hennink, W. E., and Storm, G. (2012). Drug targeting to tumors: principles, pitfalls and (pre-) clinical progress. *J. Control Release*. 161, 175–187. doi: 10.1016/j.jconrel.2011.09.063

Laroui, H., Rakhya, P., Xiao, B., Viennois, E., and Merlin, D. (2013). Nanotechnology in diagnostics and therapeutics for gastrointestinal disorders. *Dig. Liver Dis*. 45, 995–1002. doi: 10.1016/j.dld.2013.03.019

Larsson, M., Huang, W. T., Liu, D. M., and Losic, D. (2017). Local co-administration of gene-silencing RNA and drugs in cancer therapy: State-of-the art and therapeutic potential. *Cancer Treat. Rev*. 55, 128–135. doi: 10.1016/j.ctrv.2017.03.004

Liu, Y., Hardie, J., Zhang, X., and Rotello, V. M. (2017). Effects of engineered nanoparticles on the innate immune system. *Semin. Immunol*. 34, 25–32. doi: 10.1016/j.smim.2017.09.011

Luxenhofer, R., Barz, M., and Schillmeier, M. (2014). Quo vadis nanomedicine? *Nanomedicine* 9, 2083–2086. doi: 10.2217/nnm.14.140

Lv, H., Zhang, S., Wang, B., Cui, S., and Yan, J. (2006). Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Control Release*. 114, 100–109. doi: 10.1016/j.jconrel.2006.04.014

Lyass, O., Uziely, B., Ben-Yosef, R., Tzemach, D., Heshing, N. I., Lotem, M., et al. (2000). Correlation of toxicity with pharmacokinetics of pegylated liposomal doxorubicin (Doxil) in metastatic breast carcinoma. *Cancer* 89, 1037–1047. doi: 10.1002/1097-0142(20000901)89:5<1037::AID-CNCR13>3.0.CO;2-Z

Maeda, H. (2015). Toward a full understanding of the EPR effect in primary and metastatic tumors as well as issues related to its heterogeneity. *Adv. Drug Deliv. Rev*. 91, 3–6. doi: 10.1016/j.addr.2015.01.002

Maeda, H., Nakamura, H., and Fang, J. (2013). The EPR effect for macromolecular drug delivery to solid tumors: improvement of tumor uptake, lowering of systemic toxicity, and distinct tumor imaging *in vivo*. *Adv. Drug. Deliv. Rev*. 65, 71–79. doi: 10.1016/j.addr.2012.10.002

Maiseyeu, A., Mihai, G., Kampfrath, T., Simonetti, O. P., Sen, C. K., Roy, S., et al. (2009). Gadolinium-containing phosphatidylserine liposomes for molecular imaging of atherosclerosis. *J. Lipid. Res*. 50, 2157–2163. doi: 10.1194/jlr.M800405-JLR200

Mastrobattista, E., Koning, G. A., and Storm, G. (1999). Immunoliposomes for the targeted delivery of antitumor drugs. *Adv. Drug. Deliv. Rev*. 40, 103–127. doi: 10.1016/S0169-409X(99)00043-5

Matsumura, Y., and Maeda, H. (1986). A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res*. 46 (12 Pt 1), 6387–6392.

Metselaar, J. M., Van den Berg, W. B., Holthuysen, A. E., Wauben, M. H., Storm, G., and Van Lent, P. L. (2004). Liposomal targeting of glucocorticoids to synovial lining cells strongly increases therapeutic benefit in collagen type II arthritis. *Ann. Rheum. Dis*. 63, 348–353. doi: 10.1136/ard.2003.009944

Metselaar, J. M., Wauben, M. H., Wagenaar-Hilbers, J. P., Boerman, O. C., and Storm, G. (2003). Complete remission of experimental arthritis by joint targeting of glucocorticoids with long-circulating liposomes. *Arthritis Rheum*. 48, 2059–66. doi: 10.1002/art.11140

Milane, L. S., and Amiji, M. (2017). *Nanomedicine for Inflammatory Diseases*. New York, NY: CRC Press

Min, Y., Caster, J. M., Eblan, M. J., and Wang, A. Z. (2015). Clinical translation of nanomedicine. *Chem. Rev*. 115, 11147–11190. doi: 10.1021/acs.chemrev.5b00116

Mishra, D. K., Balekar, N., and Mishra, P. K. (2017). Nanoengineered strategies for siRNA delivery: from target assessment to cancer therapeutic efficacy. *Drug. Deliv. Transl. Res*. 7, 346–358. doi: 10.1007/s13346-016-0352-5

Moghimi, S. M., and Hunter, A. C. (2001). Capture of stealth nanoparticles by the body's defences. *Crit. Rev. Ther. Drug Carrier Syst*. 18, 527–550. doi: 10.1615/CritRevTherDrugCarrierSyst.v18.i6.30

Mura, S., and Couvreur, P. (2012). Nanotheranostics for personalized medicine. *Adv. Drug Deliv. Rev*. 64, 1394–1416. doi: 10.1016/j.addr.2012.06.006

Murday, J. S., Siegel, R. W., Stein, J., and Wright, J. F. (2009). Translational nanomedicine: status assessment and opportunities. *Nanomedicine* 5, 251–273. doi: 10.1016/j.nano.2009.06.001

Nakamura, H., Fang, J., and Maeda, H. (2015). Development of next-generation macromolecular drugs based on the EPR effect: challenges and pitfalls. *Expert Opin Drug Deliv*. 12, 53–64. doi: 10.1517/17425247.2014.955011

Narang, A. S., Chang, R. K., and Hussain, M. A. (2013). Pharmaceutical development and regulatory considerations for nanoparticles and nanoparticulate drug delivery systems. *J. Pharm. Sci*. 102, 3867–3882. doi: 10.1002/jps.23691

Needham, D., Anyarambhatla, G., Kong, G., and Dewhirst, M. W. (2000). A new temperature-sensitive liposome for use with mild hyperthermia: characterization and testing in a human tumor xenograft model. *Cancer Res*. 60, 1197–1201.

Nehoff, H., Parayath, N. N., Domanovitch, L., Taurin, S., and Greish, K. (2014). Nanomedicine for drug targeting: strategies beyond the enhanced permeability and retention effect. *Int. J. Nanomedicine* 9, 2539–2555. doi: 10.2147/IJN.S47129

Nel, A., Xia, T., Meng, H., Wang, X., Lin, S., Ji, Z., et al. (2013). Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. *Acc. Chem. Res*. 46, 607–621. doi: 10.1021/ar300022h

Nichols, J. W., and Bae, Y. H. (2014). EPR: evidence and fallacy. *J. Control Release*. 190, 451–464. doi: 10.1016/j.jconrel.2014.03.057

Nystrom, A. M., and Fadeel, B. (2012). Safety assessment of nanomaterials: implications for nanomedicine. *J. Control Release*. 161, 403–408. doi: 10.1016/j.jconrel.2012.01.027

Ojha, T., Pathak, V., Shi, Y., Hennink, W. E., Moonen, C. T. W., Storm, G., et al. (2017). Pharmacological and physical vessel modulation strategies to improve EPR-mediated drug targeting to tumors. *Adv. Drug Deliv. Rev*. 119, 44–60. doi: 10.1016/j.addr.2017.07.007

Oomen, A. G., Bos, P. M., Fernandes, T. F., Hund-Rinke, K., Boraschi, D., Byrne, H. J., et al. (2014). Concern-driven integrated approaches to nanomaterial testing and assessment–report of the NanoSafety Cluster Working Group 10. *Nanotoxicology* 8, 334–348. doi: 10.3109/17435390.2013.802387

Oude Blenke, E., Mastrobattista, E., and Schiffelers, R. M. (2013). Strategies for triggered drug release from tumor targeted liposomes. *Expert. Opin. Drug Deliv*. 10, 1399–1410. doi: 10.1517/17425247.2013.805742

Paradise, J., Wolf, S. M., Kuzma, J., Kuzhabekova, A., Tisdale, A. W., Kokkoli, E., et al. (2009). Developing, U.S. oversight strategies for nanobiotechnology: learning from past oversight experiences. *J. Law Med. Ethics*. 37, 688–705. doi: 10.1111/j.1748-720X.2009.00441.x

Park, J. W., Hong, K., Kirpotin, D. B., Colbern, G., Shalaby, R., Baselga, J., et al. (2002). Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery. *Clin. Cancer Res.* 8, 1172–1181.

Park, K. (2017). The drug delivery field at the inflection point: Time to fight its way out of the egg. *J. Control Release*. 267, 2–14. doi: 10.1016/j.jconrel.2017.07.030

Poste, G., Papahadjopoulos, D., and Vail, W. J. (1976). Lipid vesicles as carriers for introducing biologically active materials into cells. *Methods Cell Biol*. 14, 33–71. doi: 10.1016/S0091-679X(08)60468-9

Puri, A., Loomis, K., Smith, B., Lee, J. H., Yavlovich, A., Heldman, E., et al. (2009). Lipid-based nanoparticles as pharmaceutical drug carriers: from concepts to clinic. *Crit. Rev. Ther. Drug Carrier Syst*. 26, 523–580. doi: 10.1615/CritRevTherDrugCarrierSyst.v26.i6.10

Rahman, A. M., Yusuf, S. W., and Ewer, M. S. (2007). Anthracycline-induced cardiotoxicity and the cardiac-sparing effect of liposomal formulation. *Int. J. Nanomedicine* 2, 567–583.

Riehemann, K., Schneider, S. W., Luger, T. A., Godin, B., Ferrari, M., and Fuchs, H. (2009). Nanomedicine–challenge and perspectives. *Angew. Chem. Int. Ed. Engl.* 48, 872–897. doi: 10.1002/anie.200802585

Rizzo, L. Y., Theek, B., Storm, G., Kiessling, F., and Lammers, T. (2013). Recent progress in nanomedicine: therapeutic, diagnostic and theranostic applications. *Curr. Opin. Biotechnol.* 24, 1159–1166. doi: 10.1016/j.copbio.2013.02.020

Sainz, V., Conniot, J., Matos, A. I., Peres, C., Zupancic, E., Moura, L., et al. (2015). Regulatory aspects on nanomedicines. *Biochem. Biophys. Res. Commun.* 468, 504–510, doi: 10.1016/j.bbrc.2015.08.023

Satalkar, P., Elger, B. S., and Shaw, D. M. (2015). Defining Nano, Nanotechnology and Nanomedicine: Why Should It Matter? *Sci. Eng. Ethics* 22, 1255–1276. doi: 10.1007/s11948-015-9705-6

Sawant, R. R., and Torchilin, V. P. (2012). Challenges in development of targeted liposomal therapeutics. *AAPS J.* 14, 303–315. doi: 10.1208/s12248-012-9330-0

Senior, J. H. (1987). Fate and behavior of liposomes *in vivo:* a review of controlling factors. *Crit. Rev. Ther. Drug Carrier Syst.* 3, 123–193.

Sercombe, L., Veerati, T., Moheimani, F., Wu, S. Y., Sood, A. K., and Hua, S. (2015). Advances and challenges of liposome assisted drug delivery. *Front. Pharmacol.* 6:286. doi: 10.3389/fphar.2015.00286

Sessa, G., and Weissmann, G. (1968). Phospholipid spherules (liposomes) as a model for biological membranes. *J. Lipid. Res.* 9, 310–318.

Shajari, N., Mansoori, B., Davudian, S., Mohammadi, A., and Baradaran, B. (2017). Overcoming the challenges of siRNA delivery: nanoparticle strategies. *Curr. Drug. Deliv*. 14, 36–46. doi: 10.2174/1567201813666160816105408

Shi, J., Kantoff, P. W., Wooster, R., and Farokhzad, O. C. (2017). Cancer nanomedicine: progress, challenges and opportunities. *Nat. Rev. Cancer* 17, 20–37. doi: 10.1038/nrc.2016.108

Storm, G., Oussoren, C., Peeters, P., and Barenholz, C. (1993). "Tolerability of liposomes *in vivo*," in *Liposome Technology. 3*, ed G. Gregoriadis (Florida: CRC Press), 345–83.

Storm, G., ten Kate, M. T., Working, P. K., and Bakker-Woudenberg, I. A. (1998). Doxorubicin entrapped in sterically stabilized liposomes: effects on bacterial blood clearance capacity of the mononuclear phagocyte system. *Clin. Cancer Res.* 4, 111–115.

Svenson, S. (2012). Clinical translation of nanomedicines. *Curr. Opin. Solid. State Mater. Sci.* 16, 287–294. doi: 10.1016/j.cossms.2012.10.001

Szebeni, J. (2005). Complement activation-related pseudoallergy: a new class of drug-induced acute immune toxicity. *Toxicology* 216, 106–121. doi: 10.1016/j.tox.2005.07.023

Szebeni, J., and Barenholz, Y. (2009). "Adverse immune effects of liposomes: complement activation, immunogenicity and immune suppression," in *Harnessing biomaterials for nanomedicine: preparation, toxicity and applications*, ed P. S. Publishing (Singapore: Pan Stanford Publishing), 1–19.

Szebeni, J., and Moghimi, S. M. (2009). Liposome triggering of innate immune responses: a perspective on benefits and adverse reactions. *J. Liposome Res.* 19, 85–90. doi: 10.1080/08982100902792855

Szebeni, J., and Storm, G. (2015). Complement activation as a bioequivalence issue relevant to the development of generic liposomes and other nanoparticulate drugs. *Biochem. Biophys. Res. Commun*. 468, 490–497. doi: 10.1016/j.bbrc.2015.06.177

Talekar, M., Tran, T. H., and Amiji, M. (2015). Translational nano-medicines: targeted therapeutic delivery for cancer and inflammatory diseases. *AAPS J*. 17, 813–827. doi: 10.1208/s12248-015-9772-2

Teli, M. K., Mutalik, S., and Rajanikant, G. K. (2010). Nanotechnology and nanomedicine: going small means aiming big. *Curr. Pharm. Des.* 16, 1882–1892. doi: 10.2174/138161210791208992

Theek, B., Gremse, F., Kunjachan, S., Fokong, S., Pola, R., Pechar, M., et al. (2014). Characterizing EPR-mediated passive drug targeting using contrast-enhanced functional ultrasound imaging. *J. Control Release*. 182, 83–89. doi: 10.1016/j.jconrel.2014.03.007

Tinkle, S., McNeil, S. E., Muhlebach, S., Bawa, R., Borchard, G., Barenholz, Y. C., et al. (2014). Nanomedicines: addressing the scientific and regulatory gap. *Ann. N. Y. Acad. Sci*. 1313, 35–56. doi: 10.1111/nyas.12403

Torchilin, V. P. (2006). Multifunctional nanocarriers. *Adv. Drug Deliv. Rev*. 58, 1532–1555. doi: 10.1016/j.addr.2006.09.009

van der Meel, R., Vehmeijer, L. J., Kok, R. J., Storm, G., and van Gaal, E. V. (2013). Ligand-targeted particulate nanomedicines undergoing clinical evaluation: current status. *Adv. Drug Deliv. Rev*. 65, 1284–1298. doi: 10.1016/j.addr.2013.08.012

Wagner, V., Dullaart, A., Bock, A. K., and Zweck, A. (2006). The emerging nanomedicine landscape. *Nat. Biotechnol.* 24, 1211–1217. doi: 10.1038/nbt1006-1211

Willis, M., and Forssen, E. (1998). Ligand-targeted liposomes. *Adv. Drug Deliv. Rev*. 29, 249–271. doi: 10.1016/S0169-409X(97)00083-5

Zhang, L., Gu, F. X., Chan, J. M., Wang, A. Z., Langer, R. S., and Farokhzad, O. C. (2008). Nanoparticles in medicine: therapeutic applications and developments. *Clin. Pharmacol. Ther*. 83, 761–769. doi: 10.1038/sj.clpt.6100400

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The Advances and Challenges in Utilizing Exosomes for Delivering Cancer Therapeutics

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Nanotechnology plays an important role in advancing treatment and diagnosis of a variety of human diseases. The use of nanocarriers often leads to better pharmacokinetic and safety profiles as well as enhanced bioavailability of the entrapped molecules. Several nanoparticle formulations have already been approved by Food and Drug Administration [Doxil (1995), onivyde (1996), Abraxane (2005)] or have advanced into clinical trials (Andre et al., 2002; Von Hoff et al., 2016; Subbiah et al., 2018). These particles are typically synthesized using lipids or polymers as these materials offer significant protection against degradation from serum nucleases and proteases. Targeting ligands can also be attached to the surface of these systems with ease to enable targeted delivery. However, the reliance of single targeting ligand may not be suitable for treatment of cancer as cancer cells could quickly adapt and change surface receptor expression profiles (Wu et al., 2014). To overcome this problem, recent research has focused on using naturally occurring exosomes to deliver therapeutic cargos. Exosomes, being natural transporters, offer significant advantage for cancer treatment as the surface of exosomes are decorated with numerous ligands that can be beneficial for preferential tumor targeting. Expression of selected ligands can also be enriched through molecular engineering (Alvarez-Erviti et al., 2011).

Certain types of exosomes have been demonstrated to have higher drug delivery efficiency when compared to commonly used nanocarriers (Kim et al., 2016), thus making them ideal candidates for delivering cancer therapeutics. Due to their favorable characteristics including superior targeting capability and safety profile, they are now being investigated as an emerging class of cancer therapeutics in several clinical trials with two trials already entering phase II testing (Besse et al., 2016) (NCT01854866). For instance, dendritic cell (DC)-derived exosomes loaded with tumor antigens have been used to vaccinate cancer patients with the goal of enhancing anti-tumor immune response (Escudier et al., 2005; Morse et al., 2005; Besse et al., 2016). While enhancing T cell response is yet to be achieved in these clinical studies, significant improvement in NK cell activity was observed (Morse et al., 2005; Besse et al., 2016). In addition to targeting immune cells, exosomes have also been used to target tumor cells directly (Kamerkar et al., 2017). The ideal delivery characteristic of exosomes is, in part, due to their surface protein expression profile (e.g., CD47), which allows evasion from phagocytosis by circulating monocytes (Kim et al., 2012; Kaur et al., 2014; Kamerkar et al., 2017). As the result, exosomal nanoparticles have increased circulatory half-life that is beneficial for tumor-targeting (Kim et al., 2016). Despite the promise, the development of exosomal delivery system is still in its infancy, with three major problems requiring further investigation: (1) isolation and purification of exosomes, (2) drug and antigen loading into exosomes, and (3) delivery of cargos to target cells. Here, we discuss recent progress in overcoming these challenges (**Figure 1**).

Lack of an efficient standardized isolation and purification method is a major challenge for bringing exosome technology into the clinic. It has been reported that exosomes can be isolated and purified by single or combinations of different methods, including immunoaffinity capture, size exclusion, polymeric precipitation, ultracentrifugation, microfluidics techniques, and

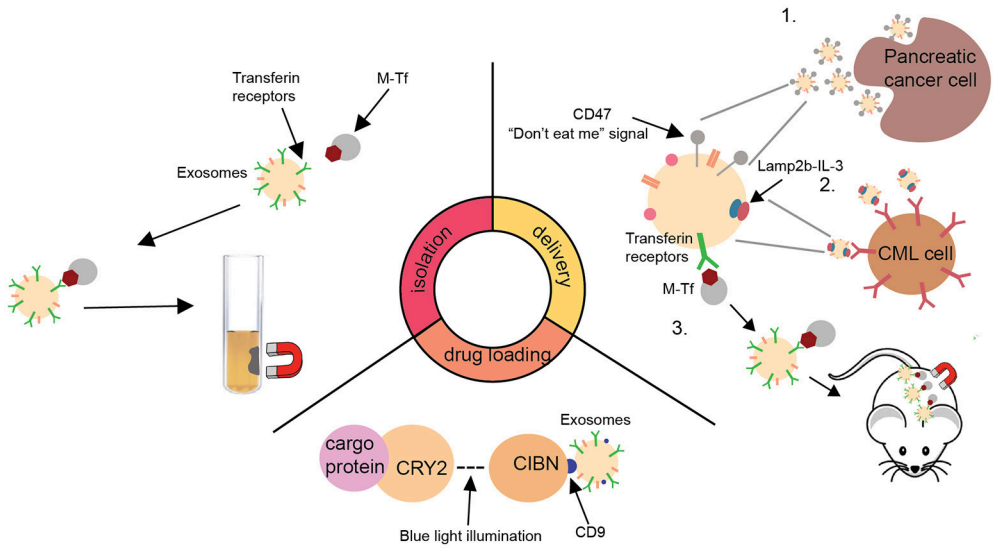

FIGURE 1 | A schematic summary of recent advances in the development of exosome delivery system. Isolation: New exosome isolation method utilizing transferrin-coated superparamagnetic (M-Tf) nanoparticles (Qi et al., 2016). Drug loading: A optically reversible protein-protein interaction (EXPLORs) technology to load proteins into exosomes (Yim et al., 2016). Delivery: Summary of three recent advances in facilitating the delivery of exosomes to tumors (Qi et al., 2016; Bellavia et al., 2017; Kamerkar et al., 2017). CML, chronic myeloid leukaemia; CRY2, cryptochrome 2; CIBN, truncated cryptochrome-interacting basic-helix-loop-helix1; Lamp2b, lysosome-associated membrane protein 2; IL-3, interleukin 3.

commercially available kits (Kim et al., 2016; Yim et al., 2016; Bellavia et al., 2017; Kamerkar et al., 2017). Combination of ultrafiltration and ultracentrifugation techniques has been used to generate clinical grade exosomes (Lamparski et al., 2002). An example of this preparation procedure includes concentrating exosomes by ultrafiltrating DC culture media or ascites fluid, followed by ultracentrifugation onto a sucrose/D_2O density cushion to eliminate non-exosome proteins and to further reduce sample volume (Escudier et al., 2005; Morse et al., 2005; Dai et al., 2008; Besse et al., 2016). This isolation method resulted in generation of exosomes that can be safely administered into patients with minimal toxicity (Escudier et al., 2005). However, due to the complexity and labor intensiveness of this isolation strategy, new methods have been developed to further facilitate future clinical development of exosomal nanosystem. A recent study has described a unique approach to improve the efficiency of exosome isolation procedure. The researchers successfully isolated large number of transferrin receptor-expressing exosomes from reticulocytes, by incubating fresh serum with transferrin-coated superparamagnetic nanoparticles and separating exosomes by magnetic adhesion (Qi et al., 2016). As these exosomes exhibit superparamagnetic behavior with a strong response to an external magnetic field, they can be efficiently separated from the blood. This technology, combined with recently developed nanoscale flow cytometry (Morales-Kastresana et al., 2017), could facilitate the isolation of purified exosomes in large scale and be applied to other ligand of interest. Purification can be achieved through staining the exosomes using Carboxyfluorescein Succinimidyl Ester (CFSE) or other fluorophores followed by size exclusion chromatography and nanoFACS analysis. NanoFACS offers multi-parametric scattered light and fluorescence imaging of exosomes with high resolution and high sensitivity. Its use permits efficient assessment and enhancement of exosome purity.

In addition to developing reliable isolation and purification methods, researchers have recently developed new strategies to load cargos into exosomes. A decade ago, two independent research groups described exosomes as carriers of information and demonstrated their ability to transfer information from one cell type to another (Ratajczak et al., 2006; Valadi et al., 2007). This fundamental concept prompted many researchers to investigate the use of exosomes as a delivery system. Currently, three major types of drug loading strategies have been investigated: incubation (Qi et al., 2016; Bellavia et al., 2017), electroporation (Kamerkar et al., 2017), and sonication (Kim et al., 2016). The most widely used technique for generating cargo-containing exosomes for clinical testing is incubation. For instance, loading of antigens into exosome can be achieved through incubating antigens directly with conditioned DC-culturing media (the source of exosomes) or purified exosomes isolated from the culture media (Escudier et al., 2005; Morse et al., 2005; Besse et al., 2016). While this is a convenient method to load antigen or drug of interest into exosomes, it is hard to precisely control loading efficiency. A recent study has compared three methods of loading, namely incubation, electroporation, and sonication, using paclitaxel as a model molecule. It was shown that a loading efficiency of 29% could be achieved with the sonication approach while 1.5 and 5.3% were achieved for the incubation and electroporation methods, respectively (Kim et al., 2016). However, it must be noted that the sonication method resulted in slight particle aggregation. Thus, development of

strategies to overcome the aggregation problem along with further improvement in loading efficiency are critical for future development of exosomal nanotechnology. In addition to traditional methods of drug loading, Yim and colleagues have recently reported a novel loading approach utilizing optically reversible protein-protein interaction (EXPLORs) technology (Yim et al., 2016). The researchers conjugated cargo proteins, mCherry, to photoreceptor cryptochrome 2 (CRY2) and induced their uptake into exosomes by overexpressing tetraspanin protein CD9 conjugated CRY-interacting basic-helix-loophelix1 (CIB1) in exosomes. The interaction between CRY2 and CIB1 was facilitated by blue light illumination. Application of this technology for nucleic acids loading along with strategies to enhance the stability of the resultant particles *in vivo* would be an exciting next set of challenges.

Another area of intense research is the development of better methods to enhance targeting ability of exosomal nanoparticles for cancer treatment. By utilizing normal human foreskin fibroblast-derived exosomes, Kamerkar and colleagues recently demonstrated the ability of exosomes to efficiently deliver $Kras^{G12D}$ siRNA to target undruggable oncogenic Kras in pancreatic tumor cells *in vivo* (Kamerkar et al., 2017). This resulted in diminished oncogenic $Kras^{G12D}$ expression, suppression of cancer cell proliferation, and an increase in overall survival in a mouse model of pancreatic cancer (Kamerkar et al., 2017). It was shown that fibroblast-derived exosomes display favorable protein expression profile on their surface which enabled efficient tumor targeting. These exosomal particles could then be taken up by tumor cells via Ras-induced micropinocytosis. In addition to the presence of naturally occurring ligands on exosomes surface, other researchers have also molecularly engineered exosome-producing cells to enrich the presence of particular ligand(s) on exosome surface in order to target a specific cancer type. For instance, Lamp2b-IL-3 expressing exosome was developed to target chronic myeloid leukemia (CML) cells preferentially as they overexpress IL-3 receptors (Bellavia et al., 2017). Utilizing this molecularly engineered system, Bellavia and colleagues successfully delivered BCR-ABL siRNA to CML cells, making them more sensitive to imatinib therapy in a CML mouse model. This technology could be applied for treatment of other IL-3 receptor over-expressing cancer types, such as lymphoma and acute myeloid leukemia. In addition to molecular methods to enhance targeting ability of exosomes to metastatic tumors, mechanical methods utilizing superparamagnetic nanoparticle-entrapped exosomes in combination with magnetic field at the tumor sites have also been developed to enhance tumor targeting. Using these superparamagnetic exosomes, Qi and colleagues successfully delivered doxorubicin to suppress tumor growth in a subcutaneous mouse model of liver cancer (Qi et al., 2016). This unique technique has provided a new approach to enhance the targeting ability of exosomes to localized tumors.

In summary, recent research has made significant progress in overcoming major barriers for using exosomes as a delivery system. Exosomes are ideal systems for delivering cancer therapeutics, owing to their size, surface expression profiles, low immunogenicity, low cytotoxicity, and long-term safety. Their use has opened a new promising avenue for cancer treatment. Scaling up the production of highly targetable therapeutic exosomes that can be used off-the-shelf which does not require generation from autologous source will be the next critical challenge to bring this promising delivery technology into the clinic.

AUTHOR CONTRIBUTIONS

SW initial conceptualization of the article. MY and SW wrote the manuscript.

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REFERENCES

Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S., and Wood, M. J. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* 29, 341–345. doi: 10.1038/nbt.1807

Andre, F., Schartz, N. E., Movassagh, M., Flament, C., Pautier, P., Morice, P., et al. (2002). Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* 360, 295–305. doi: 10.1016/S0140-6736(02)09552-1

Bellavia, D., Raimondo, S., Calabrese, G., Forte, S., Cristaldi, M., Patinella, A., et al. (2017). Interleukin 3- receptor targeted exosomes inhibit *in vitro* and *in vivo* Chronic Myelogenous Leukemia cell growth. *Theranostics* 7, 1333–1345. doi: 10.7150/thno.17092

Besse, B., Charrier, M., Lapierre, V., Dansin, E., Lantz, O., Planchard, D., et al. (2016). Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC. *Oncoimmunology* 5:e1071008. doi: 10.1080/2162402X.2015.1071008

Dai, S., Wei, D., Wu, Z., Zhou, X., Wei, X., Huang, H., et al. (2008). Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. *Mol. Ther.* 16, 782–790. doi: 10.1038/mt.2008.1

Escudier, B., Dorval, T., Chaput, N., André, F., Caby, M. P., and Novault, S., et al. (2005). Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. *J. Transl. Med.* 3:10. doi: 10.1186/1479-5876-3-10

Kamerkar, S., LeBleu, V. S., Sugimoto, H., Yang, S., Ruivo, C. F., Melo, S. A., et al. (2017). Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* 546, 498–503. doi: 10.1038/nature22341

Kaur, S., Singh, S. P., Elkahloun, A. G., Wu, W., Abu-Asab, M. S., and Roberts, D. D. (2014). CD47-dependent immunomodulatory and angiogenic activities of extracellular vesicles produced by T cells. *Matrix Biol.* 37, 49–59. doi: 10.1016/j.matbio.2014.05.007

Kim, H. S., Choi, D. Y., Yun, S. J., Choi, S. M., Kang, J. W., Jung J. W., et al (2012). Proteomic analysis of microvesicles derived from human mesenchymal stem cells. *J. Proteome Res.* 11, 839–849. doi: 10.1021/pr200682z

Kim, M. S., Haney, M. J., Zhao, Y., Mahajan, V., Deygen, I., Klyachko N. L., et al. (2016). Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. *Nanomedicine* 12, 655–664. doi: 10.1016/j.nano.2015.10.012

Lamparski, H. G., Metha-Damani, A., Yao, J. Y., Patel, S., Hsu, D. H., Ruegg, C., et al. (2002). Production and characterization of clinical grade

exosomes derived from dendritic cells. *J. Immunol. Methods* 270, 211–226. doi: 10.1016/S0022-1759(02)00330-7

Morales-Kastresana, A., Telford, B., Musich, T. A., McKinnon, K., Clayborne, C., Braig, Z., et al. (2017). Labeling extracellular vesicles for nanoscale flow cytometry. *Sci. Rep.* 7:1878. doi: 10.1038/s41598-017-01731-2

Morse, M. A., Garst, J., Osada, T., Khan, S., Hobeika, A., Clay, T. M., et al. (2005). A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J. Transl. Med.* 3:9. doi: 10.1186/1479-5876-3-9

Qi, H., Liu, C., Long, L., Ren, Y., Zhang, S., Chang, X., et al. (2016). Blood exosomes endowed with magnetic and targeting properties for cancer therapy. *ACS Nano* 10, 3323–3333. doi: 10.1021/acsnano.5b06939

Ratajczak, J., Wysoczynski, M., Hayek, F., Janowska-Wieczorek, A., and Ratajczak, M. Z. (2006). Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia* 20, 1487–1495. doi: 10.1038/sj.leu.2404296

Subbiah, V., Grilley-Olson, J. E., Combest, A. J., Sharma, N., Tran, R. H., Bobe, I., et al. (2018). Phase Ib/II Trial of NC-6004 (Nanoparticle Cisplatin) plus gemcitabine in patients with advanced solid tumors. *Clin. Cancer Res.* 24, 43–51. doi: 10.1158/1078-0432.CCR-17-1114

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., and Lötvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659. doi: 10.1038/ncb1596

Von Hoff, D. D., Mita, M. M., Ramanathan, R. K., Weiss, G. J., Mita, A. C., LoRusso P. M., et al. (2016). Phase I study of PSMA-targeted docetaxel-containing nanoparticle BIND-014 in patients with advanced solid tumors. *Clin. Cancer Res.* 22, 3157–3163. doi: 10.1158/1078-0432.CCR-15-2548

Wu, S. Y., Lopez-Berestein, G., Calin, G. A., and Sood, A. K. (2014). RNAi therapies: drugging the undruggable. *Sci. Transl. Med.* 6:240ps247. doi: 10.1126/scitranslmed.3008362

Yim, N., Ryu, S. W., Choi, K., Lee, K. R., Lee, S., Choi, H., et al. (2016). Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein-protein interaction module. *Nat. Commun.* 7:12277. doi: 10.1038/ncomms12277

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REVIEW
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Biologically Targeted Magnetic Hyperthermia: Potential and Limitations

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Hyperthermia, the mild elevation of temperature to 40–43°C, can induce cancer cell death and enhance the effects of radiotherapy and chemotherapy. However, achievement of its full potential as a clinically relevant treatment modality has been restricted by its inability to effectively and preferentially heat malignant cells. The limited spatial resolution may be circumvented by the intravenous administration of cancer-targeting magnetic nanoparticles that accumulate in the tumor, followed by the application of an alternating magnetic field to raise the temperature of the nanoparticles located in the tumor tissue. This targeted approach enables preferential heating of malignant cancer cells whilst sparing the surrounding normal tissue, potentially improving the effectiveness and safety of hyperthermia. Despite promising results in preclinical studies, there are numerous challenges that must be addressed before this technique can progress to the clinic. This review discusses these challenges and highlights the current understanding of targeted magnetic hyperthermia.

Keywords: magnetic hyperthermia, targeted therapy, iron oxide nanoparticles, cancer therapy, magnetic nanoparticles

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INTRODUCTION

Hyperthermia, a treatment aimed at raising the temperature of cancerous regions of the body to 40–43°C, can induce cancer cell death by enhancing the cytotoxic effects of radiotherapy and chemotherapy (Wust et al., 2002). Extensive preclinical and clinical research into the application of hyperthermia has been conducted, with a number of randomized trials demonstrating that, when combined with radiotherapy, it has the potential to improve the outcomes of various cancers without significantly increasing toxicity (De Haas-Kock et al., 2009; Lutgens et al., 2010). Despite these promising results, hyperthermia is rarely incorporated into modern oncological management due to its ineffectiveness when applied as a single modality treatment and a lack of large phase 3 clinical trials combining hyperthermia with both standard chemotherapy and radiotherapy (van der Zee, 2002). Furthermore, a major disadvantage of conventional hyperthermia is that, in general, both malignant and non-malignant cells are equally sensitive to heating (Dewey et al., 1977; Roizin-Towle and Pirro, 1991). This is in contrast to chemotherapy or radiotherapy, which are

generally more cytotoxic toward malignant cells. As a result, there has been significant interest in the concept of "biologically targeted magnetic hyperthermia," whereby targeted magnetic iron oxide nanoparticles (MIONs) are administered intravenously in order to heat tumors under an alternating magnetic field. In this review, we will discuss the current understanding of targeted magnetic hyperthermia and the limitations that must be overcome for further progression into clinical practice.

HYPERTHERMIA AND CELL DEATH

Hyperthermia can cause cell death through a range of different mechanisms and there are no consistent differences in thermal sensitivities between malignant and non-malignant cells (Dewey et al., 1977; Roizin-Towle and Pirro, 1991). It has been shown *in vitro* that cell viability following hyperthermia treatment is heavily influenced by both the temperature and the duration of hyperthermia (**Figure 1**). Even half a degree rise in temperature can have a substantial impact on cell viability, highlighting the importance of effective and homogenous delivery of hyperthermia (Dewey et al., 1977). One of the possible mechanisms behind the reduction in cell viability is protein denaturation with subsequent activation and deactivation of several downstream pathways (van der Zee, 2002; Wust et al., 2002). Individual proteins have specific temperature thresholds for denaturation, with highly expressed proteins generally being more tolerant to heat (Leuenberger et al., 2017). Protein denaturation occurs from approximately 40°C and higher temperatures will denature a greater proportion of proteins, which may explain why the rate of cell death rises with the temperature (Lepock, 2005b). At temperatures of 40–42°C, only a small fraction of proteins is denatured, however, some of these can subsequently co-aggregate with native proteins, thereby significantly increasing the level of aggregation (Borrelli et al., 1996). It is this combination of heat-induced denaturation and subsequent co-aggregation that is thought to affect several downstream pathways including inactivation of protein synthesis, cell cycle progression and DNA repair (Dewey et al., 1977; Kampinga et al., 2004; Lepock, 2005a). Furthermore, possibly through a mechanism that is unrelated to protein denaturation, hyperthermia can have an adverse impact on the cytoskeleton, organelles, intracellular transport, and RNA processing (Richter et al., 2010). Another potential contributor to reduction in cell viability is heat-induced alterations in the plasma and subcellular organelle membranes, as well as membrane proteins (Richter et al., 2010; Mello et al., 2017).

Sufficient application of hyperthermia can result in cell death (**Figure 1**), but if cells survive several major classes of proteins will be activated leading to thermotolerance. These classes of proteins include: heat shock proteins that stabilize misfolded proteins, proteolytic enzymes that clear denatured/aggregated proteins, RNA-, and DNA-modifying proteins that repair damage, and others (Richter et al., 2010).

In addition to the responses to hyperthermia at a cellular level described above, hyperthermia may impart its effects via several additional, unique mechanisms on cell communities and these have been investigated *in vivo*. Tumors are generally associated with hypoxic and acidic environments due to poor vasculature, conditions in which cells are known to be more susceptible to hyperthermia (Gerweck et al., 1979; Eales et al., 2016). Elevated temperatures can lead to increased perfusion within the tumor, leading to greater chemotherapeutic drug delivery and higher oxygen concentrations, which in turn can sensitize tumors to radiotherapy (Song et al., 1996; Rau et al., 2000). Hyperthermia may enhance the immune response via several mechanisms, including increased migration of immune effector cells to the tumor, modulation of cell surface molecules and various pro-inflammatory cytokines, proliferation of effector cells, and increased immune cell cytotoxicity against malignant cells (Peer et al., 2010).

Despite the multitude of mechanisms by which hyperthermia can induce cell death, it is not efficient as a single agent treatment, mainly due to its poor specificity and the development of thermotolerance which may make subsequent hyperthermia treatments less effective. However, in combination with radiotherapy or chemotherapy, hyperthermia can lead to improved patient outcomes.

HYPERTHERMIA IN COMBINATION WITH RADIOTHERAPY AND CHEMOTHERAPY

In the clinic, hyperthermia can be applied to a local area, a specific region of the body or the entire body. In the past few decades, mild elevations of temperature have been achieved by various means including thermal chambers, hot water blankets, application of electromagnetic energy, perfusion of limb or body cavity with heated fluids, ultrasound and MIONs (van der Zee, 2002; Wust et al., 2002). In order to improve the efficacy, hyperthermia has often been evaluated as an adjunct treatment to enhance radiotherapy and cytotoxic chemotherapy. One way of expressing the enhancement of radiotherapy or chemotherapy is via the thermal enhancement ratio (TER), where TER is the ratio of the dose of radiation or drug alone that is required to achieve the end point to the dose of radiation or drug combined with heat to achieve the same end point (Overgaard, 1984). As an example, 60 min of hyperthermia at 42°C, can result in a TER of nearly 2 for radiotherapy, making hyperthermia one of the most potent radiosensitizers (Overgaard, 1984). Hyperthermia is thought to enhance radiotherapy via protein denaturation and the subsequent inactivation of proteins involved in DNA repair. Inactivation of DNA repair proteins, particularly those involved in excision of clustered base damage, may prevent repair of the DNA damage induced by radiotherapy, leading to increased cell death (Kampinga and Dikomey, 2001). *In vivo*, hyperthermia can prime the tumor to radiotherapy via increased vascular perfusion and oxygenation of previously radioresistant, hypoxic areas (Song et al., 2005). Both preclinical and clinical evidence indicates that the TER is highest when hyperthermia is delivered simultaneously or in close temporal proximity to radiotherapy when protein denaturation and aggregation are likely to be at their greatest (van Leeuwen et al., 2017). Furthermore, the

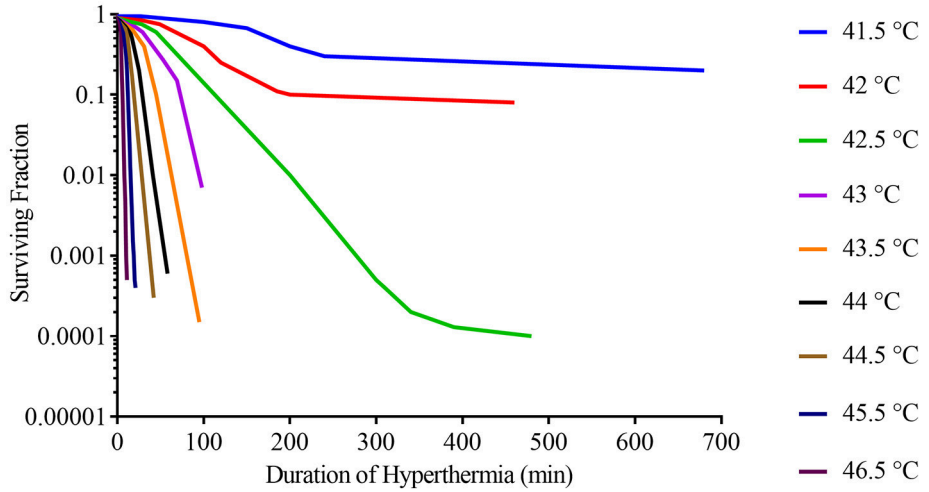

FIGURE 1 | Survival curves for asynchronous Chinese hamster ovary (CHO) cells heated at different temperatures for varying lengths of time. Adapted from Dewey et al. (1977).

TER increases with temperature and duration of hyperthermia (Overgaard, 1984).

Hyperthermia can synergistically enhance the efficacy of numerous chemotherapeutic agents including cisplatin, cyclophosphamide and bleomycin, whilst no significant enhancement for 5-fluorouracil, doxorubicin, and vincristine has been observed. For example, the application of 30 min of hyperthermia at 41.5°C *in vivo*, can result in a TER of 1.48 for cisplatin and 2.28 for cyclophosphamide (Urano et al., 1999). Although the exact mechanism for chemosensitization is poorly understood, for alkylating or alkylating-like platinum agents like cyclophosphamide and cisplatin, their ability to interact with and encourage protein denaturation may be partly responsible (Lepock, 2005b). *In vivo*, hyperthermia can lead to chemosensitivity via increased tumor blood flow and increased vascular permeability resulting in increased accumulation of chemotherapeutic agent (Song et al., 2005).

There have been a number of randomized clinical trials on the impact of hyperthermia on various cancers in combination with radiotherapy or chemotherapy or both (**Tables 1**–**3**), with many other studies currently in progress (Valdagni et al., 1988; Berdov and Menteshashvili, 1990; Datta et al., 1990; Sharma et al., 1991; Sugimachi et al., 1994; Kitamura et al., 1995; Overgaard et al., 1996; Vernon et al., 1996; Sneed et al., 1998; Harima et al., 2001; van der Zee, 2002; Jones et al., 2005; Franckena et al., 2008; Verwaal et al., 2008; Huilgol et al., 2010; Issels et al., 2010; Colombo et al., 2011; Cihoric et al., 2015; Arends et al., 2016). The majority of studies demonstrated higher rates of local response with only mild to moderate toxicities. It is worth noting that there is some heterogeneity in the outcomes, which may be due to differences in heating protocols. An area of deficiency, and perhaps one of the reasons why hyperthermia is rarely used in the clinic is that delivering sufficient hyperthermia to the tumor, whilst sparing the surrounding normal tissue, is difficult.

MAGNETIC HYPERTHERMIA

Despite the ability of hyperthermia to enhance radio- and chemotherapy treatments, toxicity due to the similar responses of malignant, and healthy tissues to hyperthermia remains a barrier to clinical application. A promising approach to overcoming this obstacle is magnetic hyperthermia, a form of hyperthermia that is currently undergoing clinical trials. It was first proposed by Gilchrist et al. (1957), who introduced the concept of injecting MIONs (20–100 nm), into lymphatic channels in order to heat residual cancer cells under an Alternating Magnetic Field (AMF) (Gilchrist et al., 1957). In 1993, Jordan et al. showed that delivering magnetic nanoparticles via direct injection into the tumor could result in much more effective and selective heating of tumors when compared to other heating techniques such as radiofrequency heating and ultrasound (Jordan et al., 1993). Furthermore, there is *in vitro* evidence that certain types of cancers including glioblastoma cells can take up magnetic nanoparticles more efficiently than non-malignant cells, although the exact mechanism is not well understood (Jordan et al., 1999). Since then, significant efforts have gone into the development of a clinical AMF system, resulting in the formation of a publicly listed company, MagForce AG based in Germany. The company has developed NanoTherm® aminosilane coated ferrofluid, NanoActivator® alternating magnetic field applicator, and NanoPlan® temperature simulation software.

In the past 2 decades, phase 1, and 2 clinical studies of intratumorally delivered magnetic nanoparticles and the subsequent application of AMF via the MagForce system have been successfully conducted for patients with glioblastoma and prostate cancers (Johannsen et al., 2005, 2007a,b; Maier-Hauff et al., 2011). Phase 1 clinical studies on patients with prostate cancer demonstrated the feasibility of the approach with no significant late treatment-related morbidity. The average temperatures achieved were in the hyperthermic

TABLE 1 | List of randomized clinical trials on hyperthermia combined with radiotherapy.

Reference	Cancer type	Number of patients randomized	Type of treatment	Outcomes	Toxicity from hyperthermia
Valdagni et al., 1988	Fixed and inoperable N3 cervical nodal squamous cell carcinoma metastases from either a previous, concomitant T1-T3 head and neck primary or unknown primary	44 nodes	Control arm: Radiotherapy Experimental arm: Radiotherapy + hyperthermia (radiative hyperthermia, 280-300 MHz within 20–25 min of irradiation, ≥42.5°C for 30 min, 2–6 treatments)	Complete response rates: 82.3% for experimental arm and 36.8% for control arm $p = 0.0152$ Thermal enhancement ratio = 2.23	Similar acute toxicities between control and experimental arm
Datta et al., 1990	Head and neck carcinoma Stage I-IV	65	Control arm: Radiotherapy Experimental arm: Radiotherapy + hyperthermia (capacitive hyperthermia, 27.12 MHz, immediately before radiotherapy, ≥42.5°C for 20 min, twice a week)	At 18 months post treatment, 19% disease free survival for control and 33% for experimental arm $p = 0.11$ For stages III and IV, control 8%, experimental 25% $p = 0.03$, 79% of study group had almost complete alleviation of pain compared to only 50% of control group $p < 0.02$	3 of 33 patients in the experimental arm developed local erythema and facial edema
Berdov and Menteshashvili, 1990	T4N0M0 Rectal carcinoma	115	Control arm: Pre-operative radiotherapy Experimental arm: Pre-operative radiotherapy and hyperthermia (capacitive hyperthermia involving an endorectal antenna, 915 MHz, 42-43°C for 1 h, 4–5 treatments, radiation delivered within 10 min)	55.4% of experimental arm were able to have an operation compared to 27.1% for control arm 5 year survival 35.6% for experimental arm compared to 6.6% for control group $p < 0.05$	Comparable post-operative complications between control and experimental arm
Sharma et al., 1991	Stage II and III Cervical Carcinoma	50	Control arm: Radiotherapy Experimental arm: Radiotherapy + hyperthermia (capacitive hyperthermia involving an intravaginal electrode, 27.12 MHz, 42-43°C for 30 min, radiation delivered within 30 min, 3 times per week for 4 weeks)	18 months of follow-up Local control 50% for control arm 70% for experimental arm $p < 0.05$	No major toxicity from hyperthermia
Perez et al., 1991	Superficial Tumors	245	Control arm: Radiotherapy Experimental arm: Radiotherapy + hyperthermia (radiative hyperthermia, 915 MHz, 43°C for 60 min immediately after irradiation, 8 treatments)	Improved local control for tumors <3cm but not for tumors >3cm	30% incidence of thermal blisters in the experimental arm
Vernon et al., 1996*	Patients with advanced primary or recurrent breast cancer having local radiotherapy rather than surgery	306	Control arm: Radiotherapy Experimental arm: Radiotherapy + hyperthermia (via various devices and frequencies depending on the study location, ≥42.5°C for ≥30 min, various intervals between radiotherapy and hyperthermia, 2-8 treatments)	Complete response for the control arm 41% 59% for hyperthermia arm $p < 0.001$ Greatest difference seen in patients with recurrent lesions in previously irradiated areas, where further irradiation was limited to low dose	More acute toxicities in the experimental arm: Blisters: 11% vs. 2% Ulceration 7% vs. 2% Necrosis 7% vs. 1% Comparable rates of late toxicity between the control and experimental arm
Overgaard et al., 1996	Recurrent or metastatic malignant melanoma	134 lesions in 70 patients	Control arm: Radiotherapy Experimental arm: Radiotherapy + hyperthermia (variable mode of delivery, hyperthermia delivered within 30 min of radiotherapy, aimed for >60 equivalent minutes of 43°C but in reality only a median of 9 equivalent minutes of 43°C achieved, 3 treatments)	Complete response rate 62% for experimental arm and 35% for radiotherapy only control arm $p = 0.003$	Similar acute or late radiation reactions in control and experimental arm

(Continued)

TABLE 1 | Continued

Reference	Cancer type	Number of patients randomized	Type of treatment	Outcomes	Toxicity from hyperthermia
Emami et al., 1996	Persistent or recurrent tumors after previous radiotherapy and/or surgery, amenable to interstitial radiotherapy	171	Control arm: Interstitial radiotherapy Experimental arm: Interstitial radiotherapy + hyperthermia (delivered by either 300-2450 MHz microwave antennas or 0.1-1 MHz radiofrequency currents, ≥43°C for 60 min, hyperthermia delivered within 60 min of irradiation, 1-2 sessions)	No difference in survival or complete response.	Similar toxicity between control and experimental arm
Van Der Zee et al., 2000	Muscle-invasive bladder cancer (including T2, T3, T4, N0, M0) Cervical Cancer Stages IIB, IIIB or IV Rectal Cancer Stage M0-M1	361	Control arm: Radiotherapy Experimental arm: Radiotherapy + hyperthermia (delivered using various systems, 42°C for 60 min, within 1–4 h after radiotherapy, 5 treatments)	Complete response rates: 39% control arm 55% experimental arm $p < 0.001$ Lower local failure rate for hyperthermia arm: (relative hazard ratio 0.76) $p = 0.04$ At 3 years, no significant difference in overall survival except for cervical cancer (51% and 27% $p = 0.009$)	Cases of burns in the experimental arm Similar rates of late radiation toxic effects between control and experimental arm
Harima et al., 2001	Stage IIIB cervical carcinoma	40	Control arm: External beam radiotherapy + high dose rate intracavitary brachytherapy Experimental arm: External beam radiotherapy + high dose rate intracavitary brachytherapy + hyperthermia (capacitive heating device, 8 MHz, delivered within 30 min of radiotherapy, for a total of 60 min, average temperature of 40.6°C achieved, 3 sessions)	Significant difference in 3-year local relapse-free survival 48.5% control arm 79.7% experimental arm $p = 0.048$ No significant improvement in 3-year overall survival and disease-free survival	Similar rates of acute or late toxicity between the control and experimental arm
Jones et al., 2005	Malignancy ≤ 3 cm in thickness from the body surface	109	All patients received hyperthermia (radiative hyperthermia, 433 MHz, for ≤1 h maximum allowable temperature of normal tissue 43°C) for 1 h. If they were unable to achieve a thermal dose of ≥0.5 CEM 43°C T90, they were not randomized. Rest of patients were then randomized. Control: No further hyperthermia but had radiotherapy Experimental: Hyperthermia + radiotherapy (twice a week, 1–2 h, targeted between 10-100 cumulative equivalent minutes at 43°C T90)	Complete response rate: Hyperthermia arm 66% Control arm 42% $p = 0.02$ Note that some patients received systemic treatment but there was no significant difference in the proportion of patients in each arm who received systemic therapy No significant difference in overall survival	Grade 1 and 2 thermal burns 41% in experimental arm 4% in control arm Grade 3 thermal burns 5% for experimental arm 2% in control arm 11% catheter (used to monitor the temperature) related side effects for experimental arm 2% for control arm
Franckena et al., 2008	Locoregionally advanced cervical cancer	114	Control arm: Radiotherapy Experimental arm: Radiotherapy + hyperthermia (via various systems depending on site, >42°C for 60 min, 5 treatments)	12 year follow-up Local control: 37% for hyperthermia arm 56% for control $p = 0.01$	Similar rates of late toxicity between control and experimental arm

(Continued)

TABLE 1 | Continued

Reference	Cancer type	Number of patients randomized	Type of treatment	Outcomes	Toxicity from hyperthermia
Huilgol et al., 2010	T2-T4, N0-N3, M0 Oropharynx, hypopharynx or oral cavity carcinoma	56	Control Arm: Radiotherapy Experimental Arm: Radiotherapy + hyperthermia (via capacitive system, 8.2MHz, power increased until patients complained of discomfort, power reduced and treatment continued for 30 min, 5-7 sessions)	Statistically significant difference in median survival of control group 145 days Experimental group 241 days	Comparable acute and late toxicities between control and experimental arm, except for overall increase in thermal burns in the experimental arm

**Meta-analysis of 5 randomized trials. The 5 trials were not published separately due to slow accrual.*

range (40–43°C), as opposed to the thermoablative range (>50°C). Although there were PSA declines following magnetic hyperthermia, responses in the monotherapy trial were of limited extent and duration and therefore, a phase 2 trial is now recruiting patients with intermediate risk prostate cancer and is evaluating magnetic hyperthermia in combination with low dose rate brachytherapy (Johannsen et al., 2010). Furthermore, MagForce has recently received an Investigational Device Exemption (IDE) for use in patients with intermediate prostate cancer undergoing active surveillance. Recruitment of patients with intermediate risk prostate cancer will commence after approval by ethics committees (Magforce, 2013, 2018). It is hoped that hyperthermia treatment in such patients can control the more aggressive component of the tumor and prevent or delay the need for radiotherapy or surgery. A phase 2 clinical trial involving 66 patients with recurrent glioblastoma, demonstrated a median overall survival of 13.4 months from the time of tumor recurrence (Maier-Hauff et al., 2011). Acute toxicities observed in this study included tachycardia (18.2%), headaches (13.6%), motor disturbances (21.2%), and convulsions (22.7%), which may be prevented with anti-epileptic drugs. In the magnetic hyperthermia study, however, no prolonged side effects were observed other than worsening motor disturbances, which may be related to disease progression rather than magnetic hyperthermia (Maier-Hauff et al., 2011). Following the phase 2 clinical trial, MagForce has been conducting a randomized, controlled trial (DRKS00005476) to determine the efficacy and safety of NanoTherm® monotherapy and NanoTherm® in combination with radiotherapy vs. radiotherapy alone in recurrent/progressive glioblastoma. The study is now closed and the final report of the data will be submitted to the official bodies this year (Magforce, 2013, 2018).

MAGNETIC IRON OXIDE NANOPARTICLES FOR MAGNETIC HYPERTHERMIA

The most commonly used materials for magnetic hyperthermia are nanometre size (10–100nm) ferrite nanoparticles, in particular magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3). Fe_3O_4 and γ-Fe_2O_3 are commonly and collectively referred to as MIONs. The magnetic properties of MIONs arise from the presence of ions with different valency in their crystal structure. For instance, Fe_3O_4 consists of two trivalent iron (III) ions and one divalent iron (II) ion. The unpaired ions result in parallel but oppositely aligned magnetic moments that do not cancel out and thus are subject to strong, spontaneous magnetization.

When exposed to an alternating magnetic field, MIONs produce heat via two main mechanisms: (1) hysteresis loss and (2) relaxational losses. Hysteresis losses occur in large MIONs which possess multiple magnetic domains. When such particles are subjected to an alternating magnetic field, the orientation of the magnetic moments will align continuously with the direction of the magnetic field as illustrated in **Figure 2**. This results in a difference in energy that is released in the form of heat (Kirschning et al., 2012). As MION size decreases, the number of magnetic domains will also decrease until a single magnetic domain remains at a threshold size of approximately 128 nm (Houlding and Rebrov, 2012). Below this size, MIONs are deemed superparamagnetic and in the presence of an AMF, heat is mainly produced by Néel relaxation and Brownian relaxation. Néel relaxation refers to rapid changes in the particle's magnetic moment when exposed to AMF (**Figure 2**). The rapid realignment is opposed by the particle's crystalline structure, resulting in heat generation. Brownian relaxation refers to the frictional heat generated from the physical rotation of particles within a supporting medium when the particles attempt to realign themselves with the changing magnetic field (**Figure 2**; Suto et al., 2009; Suriyanto et al., 2017). A more comprehensive discussion on the mechanism of heating is beyond the scope of this review and covered elsewhere (Ruta et al., 2015).

MIONs have the advantage of long term chemical stability and biocompatibility, and ease of surface modification and functionalisation when compared to other types of magnetic susceptible materials such as certain metals (e.g., iron, nickel or cobalt) or metal alloys (e.g., FePt, FeCo), (Dunn et al., 2014). Furthermore, MIONs can act as a contrast agent for computed tomography (CT) at high concentrations and magnetic resonance imaging (MRI) at lower concentrations, with several iron oxide nanoparticles previously approved by the FDA for these applications (Anselmo and Mitragotri, 2015). This is particularly useful since the concentration of the MIONs within the tumor can be estimated via CT and this can aid the estimation of hyperthermia dosimetry (Johannsen et al., 2007b). MIONs have also been shown to enhance the effects of radiotherapy even in the absence of AMF, potentially by increasing the generation

TABLE 2 | List of randomized clinical trials on hyperthermia combined with chemotherapy.

Reference	Cancer type	Number of patients randomized	Type of treatment	Outcomes	Toxicity from hyperthermia
Ghussen et al., 1984	Malignant melanoma of the extremities	107	Control arm: Local excision and regional lymph node dissection Experimental arm: Local excision and regional lymph node dissection + hyperthermia perfusion (via extracorporal heating of heparinized whole blood, limb temperatures were elevated to 42°C, 60 min) with melphalan (added once limb temperature reached ≥40°C)	Significant improvement in disease-free survival $p = 0.0001$ Significant improvement in survival. $p = 0.0207$	Higher rates of reversible post-operative complications in the experimental arm
Hafström et al., 1991	Recurrent malignant melanoma of the extremities	69	Control arm: Surgery Experimental arm: Surgery + regional hyperthermic perfusion (via extracorporeal heating of blood mixed with low molecular weight dextran and heparin, temperature of the inflow perfusate was maintained at 41.5–41.8°C, maintained for 1 h, melphalan added either beginning or at the end of hyperthermic perfusion)	Improved tumor-free survival $p = 0.044$ Difference in median survival not statistically significant	Higher rates of post-operative complications in the experimental arm
Hamazoe et al., 1994	Gastric cancer with gross serosal invasion but no gross peritoneal metastasis	82	Control arm: Surgery Experimental arm: Surgery + continuous hyperthermic peritoneal perfusion with mitomycin C (after gastrectomy, saline containing mitomycin C was heated and infused into the peritoneal cavity via silicon tubes, inflow termperature was maintained between 44–45°C, 50–60 min)	No statistically significant difference in overall survival.	Higher rates of transient abnormal blood profiles after surgery in the experimental arm
Sugimachi et al., 1994	Thoracic esophageal squamous cell carcinoma	40	Control arm: Chemotherapy +/- Oesophagectomy Experimental arm: + hyperthermia (via capacitive system involving an endotract electrode, 42.5–44°C for 30 min, 6 sessions) +/− Oesophagectomy	Subjective improvement of dysphagia: 40% in control arm vs. 70% for experimental arm Radiographic improvement: 25% in control arm and 50% in experimental arm Histological response: 18.8% in control arm vs. 58.3% in experimental arm $p < 0$ 0.05	Similar rates of toxicity between control and experimental arm
Koops et al., 1998	Primary cutaneous melanoma at high risk of having regional micrometastases	832	Control: Wide excision Experimental arm: Wide excision and isolated limb perfusion with melphalan and mild hyperthermia (limb was perfused heated perfusate, maintaining tissue temperatures of 39–40°C for 60 min, melphalan delivered once subcutaneous temperature reached 38°C)	No survival benefit	Higher rates of transient post-operative toxicity in the experimental arm
Verwaal et al., 2008	Peritoneal carcinomatosis of colorectal cancer	105	Control: Chemotherapy (5-fluorouracil, leucovorin weekly for 26 weeks or until progression or unacceptable toxicity. If treated with 5-fluorouracil within 12 months before randomization, received irinotecan at 3 weekly intervals for 6 months, or until progression or intolerable toxicity) + surgery (only if symptoms of intestinal obstruction). Experimental arm: Cytoreductive surgery, intra-operative hyperthermic intraperitoneal chemotherapy (initial warming via >3 l isotonic dialysis fluid, at 1–2 l/min and an inflow temperature of 41–42°C for 90 min, Mitomycin C added once abdominal temperature stable at 40°C) + adjuvant systemic chemotherapy.	Median follow-up of almost 8 years Median progression-free survival: 7.7 months for control arm and 12.6 months in hyperthermia arm $p = 0.02$ Median disease-specific survival: 12.6 months in control arm and 22.2 months in hyperthermia arm $p = 0.028$	Toxicity higher for experimental arm including 3 of 54 patients in the experimental arm dying from abdominal sepsis

(Continued)

TABLE 2 | Continued

Reference	Cancer type	Number of patients randomized	Type of treatment	Outcomes	Toxicity from hyperthermia
Colombo et al., 2011	Intermediate to high-risk non-muscle invasive bladder cancer	83	Control arm: Transurethral resection and 2 doses of mitomycin C Experimental arm: Transurethral resection and 2 doses of mitomycin C + hyperthermia (via a 915 MHz intravesical radiative hyperthermia device, median temperature of 42 ± 2°C for ≥40 min, 8 x weekly and 4 x monthly sessions)	Median follow-up 91 months 10-year disease-free survival: 53% with thermochemotherapy 15% with chemotherapy $p < 0.001$	Similar rates of acute and late toxicity between control and experimental arm
Arends et al., 2016	Intermediate to high risk non-muscle-invasive bladder cancer	190	Control Arm: Bacillus Calmette-Guerin immunotherapy Experimental arm: 6 × weekly mitomycin C + 6 × 6-weekly maintenance mitomycin C and hyperthermia (via a 915 MHz intravesical radiative hyperthermia device, 42 ± 2°C, 60 min, 6 x weekly sessions followed 6 further treatments at 6 week intervals)	24 month recurrence free survival was 81.8% in experimental arm and 64.8% in the control arm $p = 0.02$	Mitomycin C + Hyperthermia group associated with less urinary frequency, nocturia, incontinence, hematuria, fever, fatigue and arthralgia but more catheterisation difficulties, urethral strictures, bladder tissue reaction, bladder spasms, bladder pain, allergies

of reactive oxygen species (ROS) through the Fenton reaction (Huang et al., 2010; Klein et al., 2012; Khoei et al., 2014; Bouras et al., 2015). Finally, iron is an essential component of the human body and the average human adult naturally carries approximately 3.5–4 grams of iron. Consequently, unlike other inorganic nanoparticles, MIONs have been systemically delivered safely in large quantities in clinical settings (Hetzel et al., 2014). Furthermore, there is *in vitro* evidence that intracellular localized heating of ligand-decorated MIONS can lead to lysosomal damage of the target cells and induce cell death even in the absence of bulk heating (Creixell et al., 2011; Domenech et al., 2013).

MODE OF DELIVERY

MIONs can potentially be delivered to the tumor via intra-tumoral, intra-peritoneal, intra-arterial, intra-cavitary, and intravenous administration. Oral administration of MIONs is not feasible as most of the nanoparticles will be fecally excreted, owning to their large size (Chamorro et al., 2015). Intra-tumoral administration of MIONs efficiently localizes MIONs in the tumor and can result in effective heating of primary tumors such as prostate cancer. Intra-tumoral administration can result in very high concentrations of MIONs within the tumor and can remain localized in the tumor. When MIONs were directly injected to the prostate in men with localized prostate cancer, MIONs were still clearly visible on CT 6 weeks post injection, thereby allowing repeated magnetic hyperthermia treatments (Johannsen et al., 2005). In a separate post-mortem study of patients with glioblastoma who received MIONs, nanoparticles were restricted to the site of intra-tumoral injection, once again confirming a good retention profile (Van Landeghem et al., 2009). However, intra-tumoral delivery of MIONs is not practical for larger tumors with regional metastases and is more invasive than other techniques (**Figure 3**). Furthermore, poorly defined tumors like GBM may be better targeted by intravenously delivered MIONs which are less dependent on the operator for effective delivery, although penetrating the blood-brain barrier may be a challenge.

Intra-peritoneal mode of delivery is well suited to cancers that often spread to the peritoneal cavity such as ovarian, pancreatic and gastric cancers. Cancer targeting MIONs have been successfully delivered via the intra-peritoneal route and have demonstrated significant uptake by both primary and metastatic tumors in orthotopic mouse pancreatic cancer models (Gao et al., 2017). When compared to intravenous mode of delivery, the intra-peritoneal route achieved an intra-tumoral level that was 3-fold higher. The same system was able to carry chemotherapeutic drugs and significantly inhibited pancreatic tumors without systemic toxicity (Gao et al., 2017). Toraya-Brown et al. administered non-targeted MIONs intra-peritoneally in an aggressive mouse metastatic ovarian cancer model and demonstrated significant accumulation of MIONs in the tumor (Toraya-Brown et al., 2013). They determined that the non-targeted MIONs were taken up by peritoneal phagocytes and delivered to tumors. When under an AMF, MIONs generated enough heat to induce cell death within tumors. A separate mouse study determined that up to 5 mg/kg of MIONs can be safely delivered intraperitoneally although at higher levels, signs of oxidative damage were detected within the hepatic and renal tissues (Ma et al., 2012). Furthermore, monocyte/macrophage-like cells with a propensity to migrate into tumors, can be loaded with MIONs externally and injected intraperitoneally, after which the cells will direct MIONs for magnetic hyperthermia (Basel et al., 2012).

TABLE 3 | List of randomized clinical trials on hyperthermia combined with radiotherapy and chemotherapy.

Reference	Cancer type	Number of patients randomized	Type of treatment	Outcomes	Toxicity from hyperthermia
Kitamura et al., 1995	Squamous cell carcinoma of the thoracic esophagus undergoing neoadjuvant therapy	66	Control arm: Neoadjuvant chemoradiotherapy + surgery Experimental arm: Neoadjuvant hyperthermochemoradiotherapy (capacitive system involving an intraluminal applicator, 42.5–44°C at tumor surface for 30 min, 6 sessions)	Complete response 25% in experimental arm 5.9% in control arm 3 year survival 50.4% experimental arm 24.2% control arm	Details lacking No postoperative mortality in either arm
Sneed et al., 1998	Glioblastoma	79	Control arm: Radiotherapy + oral hydroxyurea + brachytherapy boost Experimental arm: Radiotherapy + oral hydroxyurea + brachytherapy boost + hyperthermia (radiative hyperthermia, 915 MHz, ≥42.5°C for 30 min, 15–30 min before and after brachytherapy)	Median survival: 76 weeks for control arm 85 weeks for hyperthermia arm $p = 0.02$	There was a trend ($p = 0.08$) toward more grade 3 or higher toxicities for the experimental arm Higher incidence of grade 1 and grade 2 neurological changes and seizures for the experimental arm
Issels et al., 2010	Localised high-risk soft-tissue sarcoma, extremity and retroperitoneal	341	Control arm: Neoadjuvant and adjuvant chemotherapy (etoposide, ifosfamide, doxorubicine) + local therapy (surgery +/- radiotherapy) Experimental arm: Neoadjuvant and adjuvant chemotherapy (etoposide, ifosfamide, doxorubicine) + local therapy (surgery +/- radiotherapy) + regional hyperthermia (radiative hyperthermia, 42°C for 60 min on day 1 and 4 of 3 weekly chemotherapy cycles, up to 8 sessions)	Median follow-up 34 months Significant improvement in local progression-free survival (hazard ratio = 0.58, $p = 0.003$) and disease-free survival (hazard ratio = 0.7, $p = 0.011$)	Increased pain, bolus pressure, skin burn in experimental arm

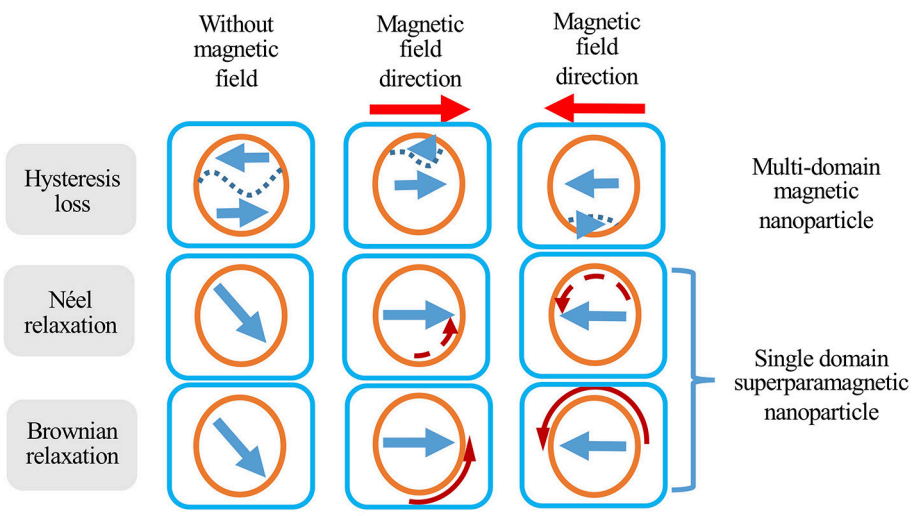

FIGURE 2 | Different heat generation mechanisms of magnetic nanoparticles in response to an alternating magnetic field. Orange circles represent MIONs, short straight arrows represent magnetic field direction, curved arrows represent the movement (solid curved arrow) or change in magnetic moment direction (dashed curved arrow), and dashed lines represent domain boundaries in multi-domain particles. Adapted from Suriyanto et al. (2017).

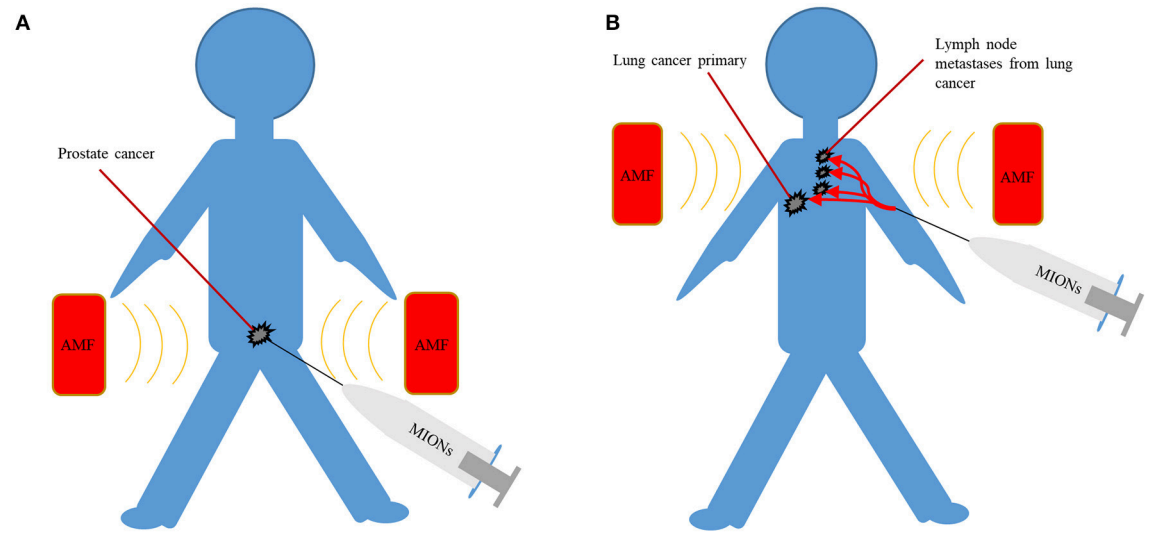

FIGURE 3 | (A) Intra-tumoral delivery can achieve high concentrations of MIONs but are only suited to localized disease such as prostate cancer. **(B)** Intravenous delivery can potentially target poorly localized malignancies, often with lymph node metastases, such as lung cancer. AMF, alternating magnetic field; MIONs, magnetic iron-oxide nanoparticles.

For bladder cancers, magnetic hyperthermia can be achieved by the direct injection of MIONs into the bladder cavity via a urinary catheter. The thick lining of the bladder will restrict the absorption of MIONs and once the treatment is completed, MIONs can be removed through the catheter, thereby minimizing systemic toxicity. The feasibility of this approach was demonstrated by Oliveira et al. in rat bladders, where temperatures of 42°C were maintained in the bladder with minimal heating of surrounding tissues (Oliveira et al., 2013).

Intra-arterial administration of chemotherapeutic drugs has been successfully applied to liver cancers in the clinic. MIONs may be well suited to this task as they tend to accumulate in the liver via the reticuloendothelial system. With this in mind, the arterial delivery of iron oxide nanoparticles has been explored in preclinical models by several investigators (Lee et al., 2013, 2017; Kim et al., 2016). In rabbits, when MIONs were delivered with iodized oil and doxorubicin, there was an increased intra-tumoral accumulation of drugs and consequently, reduced numbers of viable tumor cells (Lee et al., 2013). For lung cancer, there have been early investigations into the potential formulation of aerosolized MIONs and their delivery via a combination of a nebulizer and a magnet (Dames et al., 2007; Tewes et al., 2014; Graczyk et al., 2015). However, it would be quite challenging to deliver sufficient quantities of MIONs for the application of magnetic hyperthermia using this approach.

Although the modes of administration mentioned so far are well suited to particular scenarios, intravenous administration is the most versatile method of delivery for the widest range of cancers. Not surprisingly, intravenous delivery is one of the most common routes of administration of chemotherapeutic drugs and in the past, FDA-approved MION MRI contrast agents have been delivered intravenously. (**Figure 3**). When MIONs are delivered in this manner, the accumulation of nanoparticles within the tumor depends in part on the enhanced permeability and retention (EPR) effect (Iyer et al., 2006). The EPR effect refers to the tendency of nanoparticles to preferentially accumulate within tumors due to their leaky vasculatures and poor lymphatic drainage. Once nanoparticles have reached the tumor, targeting ligands, such as small molecules, peptides or antibodies, bound to MIONs may lead to increased association and uptake of nanoparticles by malignant cells (DeNardo et al., 2007; Balivada et al., 2010). Their preferential accumulation within the malignant cells can lead to targeted heating of tumors and sparing of adjacent normal tissue under AMF (DeNardo et al., 2007; Balivada et al., 2010). Such approaches may potentially result in more homogeneous delivery of MIONs to the tumor and would be far less operator dependent when compared to other forms of targeting. In addition, the avoidance of the surgical morbidity associated with intra-tumoral injection of MIONs in the tumor may be attractive.

So far, preclinial *in vivo* studies (**Table 4**) have been performed in order to demonstrate the concept of biologically targeted magnetic hyperthermia (**Table 4**). Huang et al. intravenously injected extremely large quantities (1,700 mg Fe/kg) of untargeted MIONs into mice and achieved a subcutaneous tumor concentration of 1.9 mg Fe/kg of tumor. Despite the lack of targeting moiety, they were able to achieve a tumor to surrounding non-tumor concentration ratio of more than 16:1 via the enhanced permeability and retention effect alone. Application of AMF achieved significant tumor control when compared to either nanoparticles or AMF alone. In the same study, mice injected with even higher doses of nanoparticles (3,400 mg Fe/kg) survived more than 12 months without showing any clinical signs of toxicity (Huang and Hainfeld, 2013). Another *in vivo* study on intravenous administration of porphyrin coated MIONs demonstrated improved melanoma

tumor control under AMF (Balivada et al., 2010). A third mouse study assessing the effectiveness of MIONs conjugated to ChL6, an antibody that targets tumor-associated antigen L6, demonstrated significant tumor accumulation and breast cancer tumor growth delays (DeNardo et al., 2007).

Despite the promising findings outlined above, preclinical studies often apply field strengths, frequencies or quantities of MIONs that are beyond what is clinically feasible and thus, further research is warranted in the areas of design, delivery, and the heating of nanoparticles, to achieve clinical translation in the future. In the following sections, areas requiring further research will be highlighted.

FACTORS INFLUENCING THE EFFICACY OF BIOLOGICALLY TARGETED MAGNETIC HYPERTHERMIA

Field Strength and Frequency

Achieving and maintaining hyperthermia in the tumor is no easy task. Due to natural thermoregulatory processes, significant power must be delivered to elevate the temperature of a particular region of the body. The heating of MIONs is dependent on a variety of factors including the concentration of MIONs, frequency and the field strength. Currently, the only clinically available AMF system in the world, NanoActivator® (MagForce AG, Germany), can operate at a frequency of 100 kHz and is able to apply fields up to 18 kA/m (Jordan et al., 2001). Although higher frequencies are technically feasible, 100 kHz was chosen to minimize eddy currents and maximize the temperature differential between normal tissues and tumors containing magnetic nanoparticles (Jordan et al., 1993). Eddy currents are electrical currents that are induced within the conductor, in this case the human body, due to the changing magnetic field, as described by Faraday's law of induction. Excessive non-specific heating of normal tissues by eddy currents is the primary determinant of the maximum tolerable field strength and frequency.

At present, there is limited clinical data on the maximum tolerable field strength and frequency. In 1984, Atkinson et al. designed a single-turn induction coil for interstitial magnetic seed therapy and conducted experiments on thoraces of numerous volunteers. They found that field intensities up to 35.8 A/m at a frequency of 13.56 MHz can be tolerated for extended periods of time. Based on this study, the assumption was made that the product of field strength and frequency should not exceed 4.85×10^8 A/m·s (Atkinson et al., 1984). However, this is not an absolute limit and in certain scenarios, this limit may be exceeded (Dutz and Hergt, 2013; Obaidat et al., 2015). In phase 1 and 2 trials of the MagForce system, using lower frequencies of 100 kHz, patients with glioblastoma were able to tolerate up to 13.5 kA/m (1.35×10^9 A/m·s or a median value of 8.5×10^8 A/m·s) whilst patients with prostate cancer were only able to tolerate up to 5 kA/m (5×10^8 A/m·s) due to discomfort in the groin and/or perineal regions (Johannsen et al., 2007a; Maier-Hauff et al., 2007; Nieskoski and Trembly, 2014). This may have been due to boundary effects between tissues of different dielectric constants and conductivity, as well as narrowing of current path in the skin folds such as the groin, resulting in hot spots (Johannsen et al., 2007a). The higher tolerable field strength in patients with glioblastoma is likely to be due to the smaller radius of the head compared to the pelvis or thorax in other studies. Considering that higher field strengths and frequencies will translate to improved heating of tumors, further research is required into improving the tolerable limits of magnetic field strengths and frequencies via improved surface cooling of hotspots that develop in the body, such as the groin (Johannsen et al., 2007b). Furthermore, with shorter duration of treatment, it is possible that higher magnetic field strength or frequency may be achievable. Another possible limitation to the maximum field strength that can be applied clinically relates to the technical challenges of designing and manufacturing a much larger system than the smaller systems utilized in the preclinical studies (**Table 4**; Jordan et al., 2001). It is advisable that future preclinical studies on biologically targeted magnetic hyperthermia focus on the application of clinically relevant magnetic field strength and frequency of 18 kA/m and 100 kHz currently available on the MagForce system.

Assuming that MIONs have been delivered to the target, the temperature can be adjusted by the alteration of magnetic field strength or frequency. For example, the hyperthermia system from Magforce controls the temperature by adjusting the magnetic field strength. As the effect of hyperthermia is heavily influenced by the temperature reached and for how long this is maintained, it is extremely important to accurately monitor the temperature during therapy and this has been previously achieved with an invasive catheter or specialized software based on imaging (Mahmoudi et al., 2018). Future studies must ensure that hyperthermia is delivered sufficiently by close monitoring of the tumor temperature.

DOSING AND TOXICITY OF MAGNETIC IRON OXIDE NANOPARTICLES

The rate of AMF-induced heating is highly dependent on the concentration of MIONs within the tumor. In clinical trials, up to 31.36 mg of Fe/cm^3 of tumor, in the form of MIONs, have been administered intra-tumorally in patients with glioblastoma (Maier-Hauff et al., 2011). Feraheme® (AMAG Pharmaceuticals, USA), an FDA approved iron oxide nanoparticle indicated for iron replacement, has been safely delivered intravenously in larger quantity than probably any other FDA approved inorganic nanoparticle so far and the recommended dose is 510 mg of Fe in the form of Feraheme®, followed by a second injection 3 to 8 days later. In the past, several patients have received two additional injections to a total dose of 2.02 g of Fe in the form of Feraheme® within a short period (Lu et al., 2010). In a hypothetical scenario, if 2.02 g of Fe in the form of MIONs, are intravenously administered to a patient with a 35 ml prostate tumor, and assuming that 1% of the dose would reach the tumor, this would result in only about 0.6 mg of Fe/cm^3 of tumor, far lower than what has been achieved with intra-tumoral administration. In addition, Feraheme contains approximately 3 nm iron oxide cores that are smaller than the MIONs that are typically associated with effective heating (Bullivant et al.,

TABLE 4 | *In vivo* studies of biologically targeted magnetic hyperthermia.

Reference	Field strength (kA/m)	Frequency (kHz)	Field strength x frequency (A/m•s)	Quantity of Fe delivered	Target	Targeting Mechanism	Summary of Results
Huang (Huang and Hainfeld, 2013)	38 kA/m	980 kHz	3.724×10^{10}	1700 mg/kg	Squamous Cell Carcinoma	EPR	Durable ablation of tumors in 84% of hyperthermia group compared to 0% for controls
Balivada (Balivada et al., 2010)	5 kA/m	366 kHz	1.830×10^{9}	13.30 mg/kg*	Melanoma	EPR + Porphyrins	Tumor volume was smaller in the hyperthermia group ($p<0.1$)
DeNardo (DeNardo et al., 2007)	56–113 kA/m	153 kHz	1.729×10^{10}	150 mg/kg*	Breast Cancer	EPR + Antibody targeting integral membrane glycoprotein	Tumor doubling/tripling/quadrupling times were increased significantly ($p < 0.05$) except for the group that received the lowest energy

**Assuming 20 g average weight of mice.*
EPR, enhanced permeability and retention; kA/m, kiloampere/metre; kHz, kilohertz; A/m•s, ampere/meter•second.

2013). For example, the nanoparticles used by MagForce contain a 12 nm iron oxide core surrounded by aminosilanes and larger crystal cores are likely to be associated with different toxicity profiles. In mice, Huang et al. was able to deliver much higher concentrations of MIONs (5.1 g Fe/kg) and determined an MTD_{50} value of 4.7 g Fe/kg, more than 100 times that delivered per kg in the Feraheme study (Huang and Hainfeld, 2013).

As the interaction of MIONs with their biological environment, and therefore their toxicity, varies with morphology, size, and surface modifications such as the addition of biocompatible coatings and targeting moieties, as well as the route of administration, each formulation needs to be tested thoroughly *in vitro* and *in vivo*. MIONs can mediate toxicity through several mechanisms that all have to be taken into account when evaluating their safety. Most intracellular toxicity is caused by generation of reactive oxygen species whereas *in vivo* disturbances of blood clotting, iron homeostasis and macrophage function, as well as organ toxicities, are additional considerations (Ilinskaya and Dobrovolskaia, 2013; Wu et al., 2014; Wei et al., 2016; Shah and Dobrovolskaia, 2018). A more detailed discussion of MION toxicity can be found in specialized review articles (Reddy et al., 2012; Liu et al., 2013; Arami et al., 2015).

To achieve sufficient heating via intravenous delivery of MIONs, further research is necessary to assess the tolerability of larger quantities of MIONs with bigger cores which are more suited to magnetic hyperthermia, and this will have to be finely balanced with size requirements for efficient intra-tumoral accumulation of nanoparticles.

HEATING EFFICIENCY OF MAGNETIC IRON OXIDE NANOPARTICLES

In order to minimize the quantity of iron oxide nanoparticles necessary for adequate magnetic hyperthermia, the development of nanoparticles with higher heating efficiency is desirable. The most common parameter for quantifying the heat generated via magnetic induction of MIONs is the Specific Absorption Rate (SAR). The experimental measurement of SAR is relatively simple. It typically involves suspending a known amount of MIONs in a liquid of known heat capacity. The test sample is exposed to an AMF of a specific strength and frequency, and the change in temperature is measured continuously over a period of time. The temperature measurement is carried out with fiber optic temperature probes to avoid electromagnetic interference with the measurement. The SAR is then calculated from the following equation (Kallumadil et al., 2009; Huang et al., 2012):

$$SAR = \frac{C}{m_{np}} \left(\frac{dT}{dt}\right)\Big|_{t=0}$$

where C is heat capacity of the fluid per unit mass of fluid, m_{np} is the mass of magnetic phase suspended in the fluid and dT/dt refers to the initial slope of temperature rise T, as a function of time, t.

It is important to note that SAR is a system-dependent parameter, that is, its value depends on the strength (H) and frequency (f) of the applied magnetic field. Therefore, direct comparison between measurements that are made using different field strength and frequency is not possible. A better parameter for this purpose is the Intrinsic Loss Power (ILP) which is mathematically described by the equation below (Kallumadil et al., 2009):

$$ILP = \frac{SAR}{H^2 f} = \frac{C}{H^2 f\, m_{np}} \left(\frac{dT}{dt}\right)\Big|_{t=0}$$

The ILP parameter is introduced under several key assumptions: (1) Test samples are single domain nanoparticles that heat up mainly via rotational relaxation; (2) Magnetic induction systems are of low frequencies at approximately 10^5–10^6 Hz; (3) Applied field strength is under the saturation field of the MIONs; (4) For the case of polydisperse MIONs in solution, the crystallite

polydispersity index (PDI) has to be greater than 0.1 (Rosensweig, 2002; Kallumadil et al., 2009). If these assumptions are not satisfied, the derived ILP values may not be valid. It is important to note that the published ILPs are only a guide, and the absolute values may not always be reliable due to the variability in the methods used to measure them and given the heating rates are very sensitive to factors such as polydispersity (Gonzales-Weimuller et al., 2009; Wildeboer et al., 2014). Different types of MIONs have highly variable heating properties. Kallumadil et al. found significant variations in the ILP between various commercially available MIONs, ranging from 0.15 to 3.12 nHm^2/kg. Heating rates can be influenced by several factors such as the ferrous iron content, size, hydrodynamic diameter, shape, number of cores, method of synthesis, and introduction of other metals such as Mn and Zn (Kallumadil et al., 2009; Blanco-Andujar et al., 2015; Hauser et al., 2015; Phong et al., 2017).

Due to the large number of variables, it is difficult to determine precisely how individual factors can impact the heating performance. In addition, the viscosity of the solvent and concentration of MIONs can further dictate the heating properties (Salas et al., 2014). Despite this, there are studies that do provide general insights to the relationship between the various characteristics and the heating properties. Several investigators have shown that in general, larger MIONs are more efficient at generating heat than smaller MIONs. (Gonzales-Weimuller et al., 2009; Lartigue et al., 2011; de La Presa et al., 2012; Jeun et al., 2012). For example, Lartigue et al. produced MIONs ranging from 4 to 35nm and coated them with rhamnose, a type of sugar. When heated under 168 kHz and 21 kA/m, the SAR was 0 W/g of Fe for 4 nm MIONs, 32 W/g of Fe for 10 nm MIONs, 61 W/g of Fe for 16 nm MIONs, and 76 W/g of Fe for 35 nm MIONs (Lartigue et al., 2011).

The shape of the nanoparticle can have a significant influence on the heating performance. Song et al. produced and compared the heating performance of quasi-cubical and spherical Fe_3O_4 nanoparticles under 100 kHz and 30 kA/m. Under equal concentration of Fe, the SAR for quasi-cubical nanoparticles were far superior (Song et al., 2012). Another study by Nemati et al. compared deformed cube (octopods) shaped MIONs with spherical nanoparticles of similar volume and demonstrated superior heating performance of the octopods (Nemati et al., 2016). Liu et al. produced ring shaped MIONs (nanorings) and compared the heating performance with a commercial MION called Resovist across a range of magnetic field strengths. Although the difference cannot be entirely attributed to the shape alone due to the differences in size, nanorings demonstrated superior heating performance, especially under the higher ranges of magnetic field strength (Liu et al., 2015). Consequently, magnetic hyperthermia via nanorings resulted in superior tumor control *in vivo* (Liu et al., 2015). Despite the superior heating rates of some of the oddly shaped MIONS, it is important to be aware that the shape can also influence the rate of uptake and toxicity (Hinde et al., 2017). These factors must be considered when designing nanoparticles for clinical applications.

The surface coating can have a significant impact on the heating performance of MIONs. Complete coating of MIONs with a low heat conductor such as SiO_2 shell can prevent the outflow of heat and reduce the heating efficiency (Gonzalez-Fernandez et al., 2009; Rivas et al., 2012). Furthermore, the thickness of the coating can also impact the heating efficiency. Liu *et al.* coated MIONs with polyethylene glycol (PEG) polymer of various length ranging from 2,000 to 20,000 Da and found that MIONs coated with shorter polymers generally heat better, possibly due to increased Brownian loss, improved thermal conductivity and dispersibility (Liu et al., 2012). One exception to this was the 31 nm MION which heated better when coated with longer PEG polymers. This was ascribed to potential agglomeration of the 31 nm MIONs with the shorter PEG, highlighting a delicate balance between stability and heating performance. The coating can also influence the pharmacokinetics of MIONs in the body which is an important consideration when developing MIONs for hyperthermia (Arami et al., 2015). Doping MIONs with Mg or Zn is another strategy that has resulted in nanoparticles with superior heating profiles, resulting in better tumor control *in vivo* (Jang et al., 2009).

Interestingly, one of the highest ILPs (23.41 nHm^2/kg) to have been reported in the past was on bacterially derived MIONs, which have a mean core diameter of approximately 30 nm (Hergt et al., 2005). Bacterial magnetosome-like cubic nanoparticles were later produced by Martinez-Boubeta et al. and demonstrated superior heating efficiency compared to spheroidal MIONs of similar size (Martinez-Boubeta et al., 2013). Le Fevre et al. have evaluated the effectiveness of magnetic hyperthermia via intra-tumorally delivered magnetosomes and achieved superior tumor control compared to chemically synthesized MIONs (Le Fèvre et al., 2017). Recently, Sangnier et al. demonstrated that magnetosomes can be tagged with tumor targeting peptide, arginine-glycine-aspartic acid (RGD), then administered intravenously in mice models for targeted delivery to tumors (Plan Sangnier et al., 2018). They applied photothermal therapy rather than magnetic hyperthermia as it was thought to be more effective. However, such approaches are likely to be limited for deep seated tumors in humans and thus, further work is required to evaluate its application for magnetic hyperthermia. Many other types of nanoparticles have been produced in the past for magnetic hyperthermia and more details can be found in other specialized review articles (Blanco-Andujar et al., 2017; Hedayatnasab et al., 2017). Higher heating efficiency would be highly desirable as it would reduce the quantity of nanoparticles, field strength and frequency required to induce significant heating.

TARGETING OF MIONS

Intravenously administered nanoparticles preferentially accumulate within tumors owing to their leaky vasculature and poor drainage. This EPR effect is well documented and was recently demonstrated in human tumors (Clark et al., 2016). In addition, structural and surface modification of MIONs can further increase tumor accumulation and up to ∼15.5%ID/g have been reported in the past (Xu et al., 2016).

Targeting of cancer cells with antibodies or other ligands can further improve the accumulation of nanoparticles within the tumor. MIONs conjugated to antibodies have been previously delivered to several tumor specific antigens including L6, HER-2 and PSMA for medical imaging and magnetic hyperthermia (DeNardo et al., 2007; Zhang et al., 2011; Tse et al., 2015). As mentioned earlier, one of the best examples is a study by DeNardo et al. in which MIONs conjugated to ChL6, an antibody that targets tumor-associated antigen L6, demonstrated significant tumor accumulation and breast cancer tumor growth delays under an AMF (DeNardo et al., 2007). Despite the potential for enhanced delivery, targeting can be associated with significant challenges in terms of the chemistry of conjugation and stability of ligand or antibody bound to nanoparticles. For example, MLN2704, a prostate specific antigen directed immunoconjugate for delivering chemotherapeutics to prostate cancer was associated with significant toxicity and limited activity due to deconjugation of the targeting antibody once in circulation (Milowsky et al., 2016). In a clinical trial of CALAA-01, a ligand bound nanoparticle siRNA delivery system, 21% of patients discontinued the study due to an adverse event and it was proposed that ligand instability was responsible for the undesirable toxicity (Zuckerman and Davis, 2015). Some of these limitations can be overcome by the application of bispecific antibodies that can spontaneously bind to both the poly ethylene glycol (PEG) coated nanoparticles and cancer specific antigens such as prostate specific membrane antigen (PSMA) or epidermal growth factor receptor (EGFR). Bispecific antibodies are composed of 2 separate single-chain fragment (scFv) and are smaller than whole antibodies. It can be stored in the freezer separate to the nanoparticles, thereby overcoming the stability issue. When administered with any PEGylated nanoparticles prior to or at the time of delivery, bispecific antibodies will spontaneously associate itself with PEGylated nanoparticles. Within the tumor, bispecific antibodies will bind to cancer specific antigens and keep the nanoparticle in close proximity to the target cancer cells, thereby, enhancing tumor accumulation (Howard et al., 2016).

To overcome the limitations of antibodies, MIONs can alternatively be conjugated to cancer specific peptides, glycosaminoglycans or aptamers. In order to target ovarian cancer, Taratula et al. synthesized MIONs conjugated to an ovarian cancer targeting Luteinizing Hormone-Release Hormone (LHRH) peptide. *In vitro*, LHRH peptide coating improved the ability of MIONs to associate with ovarian cancer cells and resulted in a significant reduction in cell viability under an alternating magnetic field (Taratula et al., 2013). For reduced immunogenicity, MIONs can be coated with hyaluronic acid, a biocompatible material that is naturally found in our body. Hyaluronic acid can target cancer cells via CD44 receptor, a commonly found cell surface marker in epithelial tumors and its potential role in magnetic hyperthermia has been demonstrated *in vitro* (Thomas et al., 2015). Nair et al. produced glioma targeting aptamers, composed of oligonucleotides, for conjugation with dextran coated iron oxide nanoparticles. Using the targeted MIONs, they were able to induce preferential damage to glioma cells via mechanical oscillation induced by a rotating magnetic field (Nair et al., 2010). It is possible that such nanoparticles may be applied for magnetic hyperthermia in the future.

For further enhancement of hyperthermia, MIONs can be directed toward intracellular organelles of cancer cells via conjugation of organelle targeting peptides. Peng et al. administered transferrin and nuclear targeting TAT peptide conjugated MIONs to mice and applied photothermal hyperthermia (Peng et al., 2017). When compared to transferrin conjugated MIONs, nuclear targeting MIONs demonstrated significant improvement in tumor control (Peng et al., 2017). Additional studies are required to confirm that such intracellular targeting strategies may be applicable for magnetic hyperthermia. Despite these exciting approaches to targeting, there is multitude of factors that can influence its effectiveness and a detailed evaluation can be found in specialized reviews (Rosenblum et al., 2018).

Another novel approach to improved tumor targeting is to suppress the reticuloendothelial system with drugs prior to the delivery of MIONs. For example, Abdollah et al. demonstrated that the suppression of Kupffer cells in the liver with dextran sulfate can significantly increase the circulating half-life of non-targeted MIONs by inhibiting the liver uptake (Abdollah et al., 2014). It is uncertain whether dextran sulfate suppression can also be applied in combination with ligand- or antibody-conjugated MIONs to prevent liver uptake and further research is warranted in this area.

Overall, several strategies are being evaluated in order to effectively target nanoparticles to the tumor whilst sparing normal tissue. Improved targeting will ultimately be the key to delivering sufficient quantities of MIONs for selective heating of tumors.

MAGNETICALLY TARGETED MIONS

Due to their magnetic properties, MIONs can be directed toward the tumor via a magnetic field. This can be applied in combination with targeted MIONs for effective magnetic hyperthermia. There are several notable examples of this approach. For gene therapy, MIONs have been used to direct intravenously administered silencing RNAs toward gastric tumors in mouse models under a magnetic field (Namiki et al., 2009). In a separate study, Garcia-Jimeno et al. were able to direct magnetoliposomes, with the aid of a magnetic field, toward the target and away from the liver and the spleen of mice (García-Jimeno et al., 2012).

For maximum uptake and retention in the tumor, it is important for MIONs or other nanoparticles to extravasate and reach the cancer cells. This can be achieved by disrupting the endothelial barrier with an external magnetic field. Qui et al. injected MIONs into mouse tail vein and used an external magnetic field to direct the particles into the lateral tail vein. Histological examination revealed that MIONs accumulated in the endothelial tissue. When a fluorophore was injected systemically, fluorescence signal was higher in the tail of the mice

subjected to the magnetic field and MIONs, due to a disruption of endothelial lining (Qiu et al., 2017). Combining these approaches may potentially improve the therapeutic efficacy of magnetic hyperthermia in the future.

OTHER METHODS TO IMPROVE THE IMPACT OF TARGETED MAGNETIC HEATING

Other novel methods of improving the effectiveness of magnetic hyperthermia have been explored. Espinosa et al. applied near-infrared laser irradiation (808 nm) during magnetic hyperthermia *in vivo* and demonstrated 2–5 fold improvements in heating when compared to magnetic hyperthermia alone although such approaches would be limited to surface tumors owing to the poor tissue penetration of laser irradiation (Espinosa et al., 2016).

In the past, there have been attempts to biologically enhance the effectiveness of magnetic hyperthermia with hyperthermia enhancing drugs such as the heat shock protein (HSP) 90 inhibitor Geldanomycin. When cells are heated, HSP 90 plays a key role in stabilizing proteins, thus, limiting the downstream effects of protein denaturation. Therefore, the inhibition of HSP 90 can lead to improved effectiveness of hyperthermia and reduce thermotolerance. For example, Ito et al. delivered Geldanomycin, and applied magnetic hyperthermia in a mouse melanoma model, which resulted in significant improvement in tumor control when compared to magnetic hyperthermia alone (Ito et al., 2009). This approach is particularly promising as HSP 90 inhibitors can independently enhance the effectiveness of radiotherapy, even in the absence of hyperthermia (Schilling et al., 2015).

There are many other hyperthermia enhancers that have been reported in the past but the majority of these agents have not been evaluated in combination with magnetic hyperthermia (Marchal et al., 1986). Protease inhibitors are another class of potent hyperthermia enhancers that have been evaluated *in vitro*. It is thought that the enhancement is achieved by inhibiting the clearance of denatured proteins within the cells (Zhu et al., 1995).

Another novel strategy is to combine magnetic hyperthermia with thermally sensitive liposomes. This can be achieved by creating a liposome with magnetic iron oxide cores embedded within. When an AMF is applied, the magnetic nanoparticles will trigger the release of the lipososomal contents. As AMF can be applied to a specific region of the body, this could result in targeted drug release and improved therapeutic effectiveness. For example, Yang et al. produced a CD90 targeted magnetoliposome encapsulating 17-AAG, a HSP 90 inhibitor. The magnetoliposome was able to simultaneously heat liver cancer stem-like cells and trigger the release of 17-AAG, thereby improving the effectiveness of magnetic hyperthermia (Yang et al., 2015).

CONCLUSION

Hyperthermia can lead to cell death via modulation of various cellular processes and is an effective treatment that can enhance the outcomes of radiotherapy and chemotherapy. One of the disadvantages is the lack of specificity toward malignant cells compared to healthy tissue. Systemic administration of targeted MIONs has the potential to improve the specificity of hyperthermia and improve its efficacy. However, several limitations must be resolved before this technology can progress to clinic. Future preclinical studies should focus on designing MIONs that can target and heat tumors more effectively. Furthermore, various hyperthermia enhancers should be evaluated in combination with magnetic hyperthermia, with the ultimate objective of achieving clinical feasibility.

AUTHOR CONTRIBUTIONS

DC drafted the manuscript, drew the figures and constructed the tables. DC, ML, JG, RQ, YN, FM, MJ, TD, and MK discussed the outline and critically reviewed the paper, the content, and the figures used.

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REFERENCES

Abdollah, M. R., Kalber, T., Tolner, B., Southern, P., Bear, J. C., Robson, M., et al. (2014). Prolonging the circulatory retention of SPIONs using dextran sulfate: *in vivo* tracking achieved by functionalisation with near-infrared dyes. *Faraday Discuss* 175, 41–58. doi: 10.1039/C4FD00114A

Anselmo, A. C., and Mitragotri, S. (2015). A review of clinical translation of inorganic nanoparticles. *AAPS J.* 17, 1041–1054. doi: 10.1208/s12248-015-9780-2

Arami, H., Khandhar, A., Liggitt, D., and Krishnan, K. M. (2015). *In vivo* delivery, pharmacokinetics, biodistribution and toxicity of iron oxide nanoparticles. *Chem. Soc. Rev.* 44, 8576–8607. doi: 10.1039/C5CS00541H

Arends, T. J., Nativ, O., Maffezzini, M., De Cobelli, O., Canepa, G., Verweij, F., et al. (2016). Results of a randomised controlled trial comparing intravesical chemohyperthermia with mitomycin C versus bacillus calmette-guerin for adjuvant treatment of patients with intermediate- and high-risk non-muscle-invasive bladder cancer. *Eur. Urol.* 69, 1046–1052. doi: 10.1016/j.eururo.2016.01.006

Atkinson, W. J., Brezovich, I. A., and Chakraborty, D. P. (1984). Usable frequencies in hyperthermia with thermal seeds. *IEEE Trans. Biomed. Eng.* 31, 70–75. doi: 10.1109/TBME.1984.325372

Balivada, S., Rachakatla, R. S., Wang, H., Samarakoon, T. N., Dani, R. K., Pyle, M., et al. (2010). A/C magnetic hyperthermia of melanoma mediated by iron(0)/iron oxide core/shell magnetic nanoparticles: a mouse study. *BMC Cancer* 10:119. doi: 10.1186/1471-2407-10-119

Basel, M. T., Balivada, S., Wang, H., Shrestha, T. B., Seo, G. M., Pyle, M., et al. (2012). Cell-delivered magnetic nanoparticles caused hyperthermia-mediated increased survival in a murine pancreatic cancer model. *Int. J. Nanomedicine* 7, 297–306. doi: 10.2147/IJN.S28344

Berdov, B. A., and Menteshashvili, G. Z. (1990). Thermoradiotherapy of patients with locally advanced carcinoma of the rectum. *Int. J. Hyperthermia* 6, 881–890. doi: 10.3109/02656739009140970

Blanco-Andujar, C., Ortega, D., Southern, P., Pankhurst, Q. A., and Thanh, N. T. K. (2015). High performance multi-core iron oxide nanoparticles for magnetic hyperthermia: microwave synthesis, and the role of core-to-core interactions. *Nanoscale* 7, 1768–1775. doi: 10.1039/C4NR06239F

Blanco-Andujar, C., Teran, F. J., and Ortega, D. (2017). "Current outlook and perspsectives on nanoparticle-mediated magnetic hyperthermia," in *Iron Oxide Nanoparticles for Biomedical Applications, 1st Edn.,* eds L. Sophie and L. Mahmoudi (Amsterdam: Elsevier), 197–245.

Borrelli, M. J., Lepock, J. R., Frey, H. E., Lee, Y. J., and Corry, P. M. (1996). Excess protein in nuclei isolated from heat-shocked cells results from a reduced extractability of nuclear proteins. *J. Cell Physiol.* 167, 369–379. doi: 10.1002/(SICI)1097-4652(199606)167:3<369::AID-JCP1>3.0.CO;2-N

Bouras, A., Kaluzova, M., and Hadjipanayis, C. G. (2015). Radiosensitivity enhancement of radioresistant glioblastoma by epidermal growth factor receptor antibody-conjugated iron-oxide nanoparticles. *J. Neuro-Oncol.* 124, 13–22. doi: 10.1007/s11060-015-1807-0

Bullivant, J. P., Zhao, S., Willenberg, B. J., Kozissnik, B., Batich, C. D., and Dobson, J. (2013). Materials characterization of Feraheme/ferumoxytol and preliminary evaluation of its potential for magnetic fluid hyperthermia. *Int. J. Mol. Sci.* 14, 17501–17510. doi: 10.3390/ijms140917501

Chamorro, S., Gutiérrez, L., Vaquero, M. P., Verdoy, D., Salas, G., Luengo, Y., et al. (2015). Safety assessment of chronic oral exposure to iron oxide nanoparticles. *Nanotechnology* 26:205101. doi: 10.1088/0957-4484/26/20/205101

Cihoric, N., Tsikkinis, A., Van Rhoon, G., Crezee, H., Aebersold, D. M., Bodis, S., et al. (2015). Hyperthermia-related clinical trials on cancer treatment within the ClinicalTrials.gov registry. *Int. J. Hyperthermia* 31, 609–614. doi: 10.3109/02656736.2015.1040471

Clark, A. J., Wiley, D. T., Zuckerman, J. E., Webster, P., Chao, J., Lin, J., et al. (2016). CRLX101 nanoparticles localize in human tumors and not in adjacent, nonneoplastic tissue after intravenous dosing. *Proc. Natl. Acad. Sci. U.S.A.* 113, 3850–3854. doi: 10.1073/pnas.1603018113

Colombo, R., Salonia, A., Leib, Z., Pavone-Macaluso, M., and Engelstein, D. (2011). Long-term outcomes of a randomized controlled trial comparing thermochemotherapy with mitomycin-C alone as adjuvant treatment for non-muscle-invasive bladder cancer (NMIBC). *BJU Int.* 107, 912–918. doi: 10.1111/j.1464-410X.2010.09654.x

Creixell, M., Bohorquez, A. C., Torres-Lugo, M., and Rinaldi, C. (2011). EGFR-targeted magnetic nanoparticle heaters kill cancer cells without a perceptible temperature rise. *ACS Nano* 5, 7124–7129. doi: 10.1021/nn201822b

Dames, P., Gleich, B., Flemmer, A., Hajek, K., Seidl, N., Wiekhorst, F., et al. (2007). Targeted delivery of magnetic aerosol droplets to the lung. *Nat. Nanotechnol.* 2, 495–499. doi: 10.1038/nnano.2007.217

Datta, N. R., Bose, A. K., Kapoor, H. K., and Gupta, S. (1990). Head and neck cancers: results of thermoradiotherapy versus radiotherapy. *Int. J. Hyperthermia* 6, 479–486. doi: 10.3109/02656739009140944

De Haas-Kock, D. F., Buijsen, J., Pijls-Johannesma, M., Lutgens, L., Lammering, G., Van Mastrigt, G. A., et al. (2009). Concomitant hyperthermia and radiation therapy for treating locally advanced rectal cancer. *Cochrane Database Syst. Rev.* CD006269. doi: 10.1002/14651858.CD006269.pub2

de La Presa, P., Luengo, Y., Multigner, M., Costo, R., Morales, M. P., Rivero, G., et al. (2012). Study of heating efficiency as a function of concentration, size, and applied field in Gamma-Fe2O3 nanoparticles. *J. Phys. Chem. C* 116, 25602–25610. doi: 10.1021/jp310771p

DeNardo, S. J., Denardo, G. L., Natarajan, A., Miers, L. A., Foreman, A. R., Gruettner, C., et al. (2007). Thermal dosimetry predictive of efficacy of 111In-ChL6 nanoparticle AMF–induced thermoablative therapy for human breast cancer in mice. *J. Nucl. Med.* 48, 437–444.

Dewey, W. C., Hopwood, L. E., Sapareto, S. A., and Gerweck, L. E. (1977). Cellular responses to combinations of hyperthermia and radiation. *Radiology* 123, 463–474. doi: 10.1148/123.2.463

Domenech, M., Marrero-Berrios, I., Torres-Lugo, M., and Rinaldi, C. (2013). Lysosomal membrane permeabilization by targeted magnetic nanoparticles in alternating magnetic fields. *ACS Nano* 7, 5091–5101. doi: 10.1021/nn4007048

Dunn, A. E., Dunn, D. J., Lim, M., Boyer, C., and Thanh, N. T. K. (2014). Recent developments in the design of nanomaterials for photothermal and magnetic hyperthermia induced controllable drug delivery. *Nanoscience* 2, 225–254. doi: 10.1039/9781849737623-00225

Dutz, S., and Hergt, R. (2013). Magnetic nanoparticle heating and heat transfer on a microscale: basic principles, realities and physical limitations of hyperthermia for tumour therapy. *Int. J. Hyperther.* 29, 790–800. doi: 10.3109/02656736.2013.822993

Eales, K. L., Hollinshead, K. E., and Tennant, D. A. (2016). Hypoxia and metabolic adaptation of cancer cells. *Oncogenesis* 5:e190. doi: 10.1038/oncsis.2015.50

Emami, B., Scott, C., Perez, C. A., Asbell, S., Swift, P., Grigsby, P., et al. (1996). Phase III study of interstitial thermoradiotherapy compared with interstitial radiotherapy alone in the treatment of recurrent or persistent human tumors: A prospectively controlled randomized study by the Radiation Therapy Oncology Group. *Int. J. Radiat. Oncol. Biol. Phys.* 34, 1097–1104. doi: 10.1016/0360-3016(95)02137-X

Espinosa, A., Di Corato, R., Kolosnjaj-Tabi, J., Flaud, P., Pellegrino, T., and Wilhelm, C. (2016). Duality of iron oxide nanoparticles in cancer therapy: amplification of heating efficiency by magnetic hyperthermia and photothermal bimodal treatment. *ACS Nano* 10, 2436–2446. doi: 10.1021/acsnano.5b07249

Franckena, M., Stalpers, L. J., Koper, P. C., Wiggenraad, R. G., Hoogenraad, W. J., Van Dijk, J. D., et al. (2008). Long-term improvement in treatment outcome after radiotherapy and hyperthermia in locoregionally advanced cervix cancer: an update of the Dutch Deep Hyperthermia Trial. *Int. J. Radiat. Oncol. Biol. Phys.* 70, 1176–1182. doi: 10.1016/j.ijrobp.2007.07.2348

Gao, N., Bozeman, E. N., Qian, W., Wang, L., Chen, H., Lipowska, M., et al. (2017). Tumor penetrating theranostic nanoparticles for enhancement of targeted and image-guided drug delivery into peritoneal tumors following intraperitoneal delivery. *Theranostics* 7, 1689–1704. doi: 10.7150/thno.18125

García-Jimeno, S., Escribano, E., Queralt, J., and Estelrich, J. (2012). External magnetic field-induced selective biodistribution of magnetoliposomes in mice. *Nanoscale Res. Lett.* 7: 452. doi: 10.1186/1556-276X-7-452

Gerweck, L. E., Nygaard, T. G., and Burlett, M. (1979). Response of cells to hyperthermia under acute and chronic hypoxic conditions. *Cancer Res.* 39, 966–972.

Ghussen, F., Nagel, K., Groth, W., Muller, J. M., and Stutzer, H. (1984). A prospective randomized study of regional extremity perfusion in patients with malignant melanoma. *Ann. Surg.* 200, 764–768. doi: 10.1097/00000658-198412000-00016

Gilchrist, R. K., Medal, R., Shorey, W. D., Hanselman, R. C., Parrott, J. C., and Taylor, C. B. (1957). Selective inductive heating of lymph nodes. *Ann. Surg.* 146, 596–606. doi: 10.1097/00000658-195710000-00007

Gonzales-Weimuller, M., Zeisberger, M., and Krishnan, K. M. (2009). Size-dependant heating rates of iron oxide nanoparticles for magnetic fluid hyperthermia. *J. Magn. Magn. Mater.* 321, 1947–1950. doi: 10.1016/j.jmmm.2008.12.017

Gonzalez-Fernandez, M. A., Torres, T. E., Andres-Verges, M., Costo, R., De La Presa, P., Serna, C. J., et al. (2009). Magnetic nanoparticles for power absorption: optimizing size, shape and magnetic properties. *J. Solid State Chem.* 182, 2779–2784. doi: 10.1016/j.jssc.2009.07.047

Graczyk, H., Bryan, L. C., Lewinski, N., Suarez, G., Coullerez, G., Bowen, P., et al. (2015). Physicochemical characterization of nebulized superparamagnetic iron oxide nanoparticles (SPIONs). *J. Aerosol Med. Pulm. Drug Deliv.* 28, 43–51. doi: 10.1089/jamp.2013.1117

Hafström, L., Rudenstam, C. M., Blomquist, E., Ingvar, C., Jonsson, P. E., Lagerlof, B., et al. (1991). Regional hyperthermic perfusion with melphalan after surgery for recurrent malignant-melanoma of the extremities. *J. Clin. Oncol.* 9, 2091–2094. doi: 10.1200/JCO.1991.9.12.2091

Hamazoe, R., Maeta, M., and Kaibara, N. (1994). Intraperitoneal thermochemotherapy for prevention of peritoneal recurrence of gastric cancer. Final results of a randomized controlled study. *Cancer* 73, 2048–2052. doi: 10.1002/1097-0142(19940415)73:8<2048::AID-CNCR2820730806>3.0.CO;2-Q

Harima, Y., Nagata, K., Harima, K., Ostapenko, V. V., Tanaka, Y., and Sawada, S. (2001). A randomized clinical trial of radiation therapy versus thermoradiotherapy in stage IIIB cervical carcinoma. *Int. J. Hyperthermia* 17, 97–105. doi: 10.1080/02656730010001333

Hauser, A. K., Mathias, R., Anderson, K. W., and Hilt, J. Z. (2015). The effects of synthesis method on the physical and chemical properties of dextran coated iron oxide nanoparticles. *Mater. Chem. Phys.* 160, 177–186. doi: 10.1016/j.matchemphys.2015.04.022

Hedayatnasab, Z., Abnisa, F., and Daud, W. M. A. W. (2017). Review on magnetic nanoparticles for magnetic nanofluid hyperthermia application. *Mater. Des.* 123, 174–196. doi: 10.1016/j.matdes.2017.03.036

Hergt, R., Hiergeist, R., Zeisberger, M., Schuler, D., Heyen, U., Hilger, I., et al. (2005). Magnetic properties of bacterial magnetosomes as potential diagnostic and therapeutic tools. *J. Magn. Magn. Mater.* 293, 80–86. doi: 10.1016/j.jmmm.2005.01.047

Hetzel, D., Strauss, W., Bernard, K., Li, Z., Urboniene, A., and Allen, L. F. (2014). A Phase, III, randomized, open-label trial of ferumoxytol compared with iron sucrose for the treatment of iron deficiency anemia in patients with a history of unsatisfactory oral iron therapy. *Am. J. Hematol.* 89, 646–650. doi: 10.1002/ajh.23712

Hinde, E., Thammasiraphop, K., Duong, H. T. T., Yeow, J., Karagoz, B., Boyer, C., et al. (2017). Pair correlation microscopy reveals the role of nanoparticle shape in intracellular transport and site of drug release. *Nat. Nanotechnol.* 12, 81–89. doi: 10.1038/nnano.2016.160

Houlding, T. K., and Rebrov, E. V. (2012). Application of alternative energy forms in catalytic reactor engineering. *Green Process Synth.* 1, 19–31. doi: 10.1515/greenps-2011-0502

Howard, C. B., Fletcher, N., Houston, Z. H., Fuchs, A. V., Boase, N. R. B., Simpson, J. D., et al. (2016). Overcoming instability of antibody-nanomaterial conjugates: next generation targeted nanomedicines using bispecific antibodies. *Adv. Healthc. Mater.* 5, 2055–2068. doi: 10.1002/adhm.201600263

Huang, F. K., Chen, W. C., Lai, S. F., Liu, C. J., Wang, C. L., Wang, C. H., et al. (2010). Enhancement of irradiation effects on cancer cells by cross-linked dextran-coated iron oxide (CLIO) nanoparticles. *Phys. Med. Biol.* 55, 469–482. doi: 10.1088/0031-9155/55/2/009

Huang, H. S., and Hainfeld, J. F. (2013). Intravenous magnetic nanoparticle cancer hyperthermia. *Int. J. Nanomedicine* 8, 2521–2532. doi: 10.2147/IJN.S43770

Huang, S., Wang, S. Y., Gupta, A., Borca-Tasciuc, D. A., and Salon, S. J. (2012). On the measurement technique for specific absorption rate of nanoparticles in an alternating electromagnetic field. *Meas. Sci. Technol.* 23, 1–6. doi: 10.1088/0957-0233/23/3/035701

Huilgol, N. G., Gupta, S., and Sridhar, C. R. (2010). Hyperthermia with radiation in the treatment of locally advanced head and neck cancer: a report of randomized trial. *J. Cancer Res. Ther.* 6, 492–496. doi: 10.4103/0973-1482.77101

Ilinskaya, A. N., and Dobrovolskaia, M. A. (2013). Nanoparticles and the blood coagulation system. Part II: safety concerns. *Nanomedicine* 8, 969–981. doi: 10.2217/nnm.13.49

Issels, R. D., Lindner, L. H., Verweij, J., Wust, P., Reichardt, P., Schem, B. C., et al. (2010). Neo-adjuvant chemotherapy alone or with regional hyperthermia for localised high-risk soft-tissue sarcoma: a randomised phase 3 multicentre study. *Lancet Oncol.* 11, 561–570. doi: 10.1016/S1470-2045(10)70071-1

Ito, A., Saito, H., Mitobe, K., Minamiya, Y., Takahashi, N., Maruyama, K., et al. (2009). Inhibition of heat shock protein 90 sensitizes melanoma cells to thermosensitive ferromagnetic particle-mediated hyperthermia with low Curie temperature. *Cancer Sci.* 100, 558–564. doi: 10.1111/j.1349-7006.2008.01072.x

Iyer, A. K., Khaled, G., Fang, J., and Maeda, H. (2006). Exploiting the enhanced permeability and retention effect for tumor targeting. *Drug Discov. Today* 11, 812–818. doi: 10.1016/j.drudis.2006.07.005

Jang, J. T., Nah, H., Lee, J. H., Moon, S. H., Kim, M. G., and Cheon, J. (2009). Critical Enhancements of MRI contrast and hyperthermic effects by dopant-controlled magnetic nanoparticles. *Angew. Chem. Int. Ed.* 48, 1234–1238. doi: 10.1002/anie.200805149

Jeun, M., Lee, S., Kang, J. K., Tomitaka, A., Kang, K. W., Kim, Y. I., et al. (2012). Physical limits of pure superparamagnetic Fe_3O_4 nanoparticles for a local hyperthermia agent in nanomedicine. *Appl. Phys. Lett.* 100:092406. doi: 10.1063/1.3689751

Johannsen, M., Gneveckow, U., Eckelt, L., Feussner, A., Waldofner, N., Scholz, R., et al. (2005). Clinical hyperthermia of prostate cancer using magnetic nanoparticles: presentation of a new interstitial technique. *Int. J. Hyperthermia* 21, 637–647. doi: 10.1080/02656730500158360

Johannsen, M., Gneveckow, U., Taymoorian, K., Thiesen, B., Waldofner, N., Scholz, R., et al. (2007a). Morbidity and quality of life during thermotherapy using magnetic nanoparticles in locally recurrent prostate cancer: results of a prospective phase I trial. *Int. J. Hyperthermia* 23, 315–323. doi: 10.1080/02656730601175479

Johannsen, M., Gneveckow, U., Thiesen, B., Taymoorian, K., Cho, C. H., Waldofner, N., et al. (2007b). Thermotherapy of prostate cancer using magnetic nanoparticles: feasibility, imaging, and three-dimensional temperature distribution. *Eur. Urol.* 52, 1653–1661. doi: 10.1016/j.eururo.2006.11.023

Johannsen, M., Thiesen, B., Wust, P., and Jordan, A. (2010). Magnetic nanoparticle hyperthermia for prostate cancer. *Int. J. Hyperthermia* 26, 790–795. doi: 10.3109/02656731003745740

Jones, E. L., Oleson, J. R., Prosnitz, L. R., Samulski, T. V., Vujaskovic, Z., Yu, D., et al. (2005). Randomized trial of hyperthermia and radiation for superficial tumors. *J. Clin. Oncol.* 23, 3079–3085. doi: 10.1200/JCO.2005.05.520

Jordan, A., Scholz, R., Maier-Hauff, K., Johannsen, M., Wust, P., Nadobny, J., et al. (2001). Presentation of a new magnetic field therapy system for the treatment of human solid tumors with magnetic fluid hyperthermia. *J. Magn. Magn. Mater.* 225, 118–126. doi: 10.1016/S0304-8853(00)01239-7

Jordan, A., Scholz, R., Wust, P., Fahling, H., and Felix, R. (1999). Magnetic fluid hyperthermia (MFH): cancer treatment with AC magnetic field induced excitation of biocompatible superparamagnetic nanoparticles. *J. Magn. Magn. Mater.* 201, 413–419. doi: 10.1016/S0304-8853(99)00088-8

Jordan, A., Wust, P., Fähling, H., John, W., Hinz, A., and Felix, R. (1993). Inductive heating of ferrimagnetic particles and magnetic fluids - physical evaluation of their potential for hyperthermia. *Int. J. Hyperthermia* 9, 51–68. doi: 10.3109/02656739309061478

Kallumadil, M., Tada, M., Nakagawa, T., Abe, M., Southern, P., and Pankhurst, Q. A. (2009). Suitability of commercial colloids for magnetic hyperthermia. *J. Magn. Magn. Mater.* 321, 3650–3651. doi: 10.1016/j.jmmm.2009.06.069

Kampinga, H. H., and Dikomey, E. (2001). Hyperthermic radiosensitization: mode of action and clinical relevance. *Int. J. Radiat. Biol.* 77, 399–408. doi: 10.1080/09553000010024687

Kampinga, H. H., Dynlacht, J. R., and Dikomey, E. (2004). Mechanism of radiosensitization by hyperthermia (43°C) as derived from studies with DNA repair defective mutant cell lines. *Int. J. Hyperthermia* 20, 131–139. doi: 10.1080/02656730310001627713

Khoei, S., Mahdavi, S. R., Fakhimikabir, H., Shakeri-Zadeh, A., and Hashemian, A. (2014). The role of iron oxide nanoparticles in the radiosensitization of human prostate carcinoma cell line DU145 at megavoltage radiation energies. *Int. J. Radiat. Biol.* 90, 351–356. doi: 10.3109/09553002.2014.888104

Kim, D. H., Li, W., Chen, J., Zhang, Z., Green, R. M., Huang, S., et al. (2016). Multimodal imaging of nanocomposite microspheres for transcatheter intra-arterial drug delivery to liver tumors. *Sci. Rep.* 6:29653. doi: 10.1038/srep29653

Kirschning, A., Kupracz, L., and Hartwig, J. (2012). New synthetic opportunities in miniaturized flow reactors with inductive heating. *Chem. Lett.* 41, 562–570. doi: 10.1246/cl.2012.562

Kitamura, K., Kuwano, H., Watanabe, M., Nozoe, T., Yasuda, M., Sumiyoshi, K., et al. (1995). Prospective randomized study of hyperthermia combined with chemoradiotherapy for esophageal carcinoma. *J. Surg. Oncol.* 60, 55–58. doi: 10.1002/jso.2930600111

Klein, S., Sommer, A., Distel, L. V., Neuhuber, W., and Kryschi, C. (2012). Superparamagnetic iron oxide nanoparticles as radiosensitizer via enhanced reactive oxygen species formation. *Biochem. Biophys. Res. Commun.* 425, 393–397. doi: 10.1016/j.bbrc.2012.07.108

Koops, H. S., Vaglini, M., Suciu, S., Kroon, B. B. R., Thompson, J. F., Gohl, J., et al. (1998). Prophylactic isolated limb perfusion for localized, high-risk limb melanoma: results of a multicenter randomized phase III trial. *J. Clin. Oncol.* 16, 2906–2912. doi: 10.1200/JCO.1998.16.9.2906

Lartigue, L., Innocenti, C., Kalaivani, T., Awwad, A., Duque, M. D. S., Guari, Y., et al. (2011). Water-dispersible sugar-coated iron oxide nanoparticles. An evaluation of their relaxometric and magnetic hyperthermia properties. *J. Am. Chem. Soc.* 133, 10459–10472. doi: 10.1021/ja111448t

Le Fèvre, R., Durand-Dubief, M., Chebbi, I., Mandawala, C., Lagroix, F., Valet, J. P., et al. (2017). Enhanced antitumor efficacy of biocompatible magnetosomes for the magnetic hyperthermia treatment of glioblastoma. *Theranostics* 7, 4618–4631. doi: 10.7150/thno.18927

Lee, I. J., Ahn, C. H., Cha, E. J., Chung, I. J., Chung, J. W., and Kim, Y. I. (2013). Improved drug targeting to liver tumors after intra-arterial delivery using superparamagnetic iron oxide and iodized oil: preclinical study in a rabbit model. *Invest. Radiol.* 48, 826–833. doi: 10.1097/RLI.0b013e31829c13ef

Lee, I. J., Park, J. Y., Kim, Y. I., Lee, Y. S., Jeong, J. M., Kim, J., et al. (2017). Image-based analysis of tumor localization after intra-arterial delivery of technetium-99m-labeled SPIO using SPECT/CT and MRI. *Mol. Imaging* 16:1536012116689001. doi: 10.1177/1536012116689001

Lepock, J. R. (2005a). How do cells respond to their thermal environment? *Int. J. Hyperthermia* 21, 681–687. doi: 10.1080/02656730500307298

Lepock, J. R. (2005b). Measurement of protein stability and protein denaturation in cells using differential scanning calorimetry. *Methods* 35, 117–125. doi: 10.1016/j.ymeth.2004.08.002

Leuenberger, P., Ganscha, S., Kahraman, A., Cappelletti, V., Boersema, P. J., Von Mering, C., et al. (2017). Cell-wide analysis of protein thermal unfolding reveals determinants of thermostability. *Science* 355:eaai7825. doi: 10.1126/science.aai7825

Liu, G., Gao, J. H., Ai, H., and Chen, X. Y. (2013). Applications and potential toxicity of magnetic iron oxide nanoparticles. *Small* 9, 1533–1545. doi: 10.1002/smll.201201531

Liu, X. L., Fan, H. M., Yi, J. B., Yang, Y., Choo, E. S. G., Xue, J. M., et al. (2012). Optimization of surface coating on Fe_3O_4 nanoparticles for high performance magnetic hyperthermia agents. *J. Mater. Chem.* 22, 8235–8244. doi: 10.1039/c2jm30472d

Liu, X. L., Yang, Y., Ng, C. T., Zhao, L. Y., Zhang, Y., Bay, B. H., et al. (2015). Magnetic vortex nanorings: a new class of hyperthermia agent for highly efficient *in vivo* regression of tumors. *Adv. Mater.* 27, 1939–44. doi: 10.1002/adma.201405036

Lu, M., Cohen, M. H., Rieves, D., and Pazdur, R. (2010). FDA report: ferumoxytol for intravenous iron therapy in adult patients with chronic kidney disease. *Am. J. Hematol.* 85, 315–319. doi: 10.1002/ajh.21656

Lutgens, L., Van Der Zee, J., Pijls-Johannesma, M., De Haas-Kock, D. F., Buijsen, J., Mastrigt, G. A., et al. (2010). Combined use of hyperthermia and radiation therapy for treating locally advanced cervix carcinoma. *Cochrane Database Syst. Rev.* CD006377. doi: 10.1002/14651858.CD006377.pub2

Ma, P., Luo, Q., Chen, J., Gan, Y., Du, J., Ding, S., et al. (2012). Intraperitoneal injection of magnetic Fe_3O_4-nanoparticle induces hepatic and renal tissue injury via oxidative stress in mice. *Int. J. Nanomedicine* 7, 4809–4818. doi: 10.2147/IJN.S34349

Magforce (2013). Available online at: https://www.drks.de/drks_web/navigate.do?navigationId=trial.HTML&TRIAL_ID=DRKS00005476 [Accessed *May* 29 2018].

Magforce (2018). *"Annual Report 2017: Important Cornerstones Laid for Two Pillar Strategy"*, Available online at: http://magforce.de/fileadmin/magforce/5_presse/finanzberichte/Geschaeftsbericht_2017/magforce_gb17_e_s.pdf

Mahmoudi, K., Bouras, A., Bozec, D., Ivkov, R., and Hadjipanayis, C. (2018). Magnetic hyperthermia therapy for the treatment of glioblastoma: a review of the therapy's history, efficacy and application in humans. *Int. J. Hyperthermia*. doi: 10.1080/02656736.2018.1430867.[Epub ahead of print].

Maier-Hauff, K., Rothe, R., Scholz, R., Gneveckow, U., Wust, P., Thiesen, B., et al. (2007). Intracranial thermotherapy using magnetic nanoparticles combined with external beam radiotherapy: results of a feasibility study on patients with glioblastoma multiforme. *J. Neuro-Oncol.* 81, 53–60. doi: 10.1007/s11060-006-9195-0

Maier-Hauff, K., Ulrich, F., Nestler, D., Niehoff, H., Wust, P., Thiesen, B., et al. (2011). Efficacy and safety of intratumoral thermotherapy using magnetic iron-oxide nanoparticles combined with external beam radiotherapy on patients with recurrent glioblastoma multiforme. *J. Neuro-Oncol.* 103, 317–324. doi: 10.1007/s11060-010-0389-0

Marchal, C., Anghileri, L. J., Escanye, M. C., and Robert, J. (1986). Hyperthermia and cytotoxic drugs. Possible use of lanthanum as a potentiator of hyperthermia. *Int. J. Hyperthermia* 2, 83–92. doi: 10.3109/02656738609019997

Martinez-Boubeta, C., Simeonidis, K., Makridis, A., Angelakeris, M., Iglesias, O., Guardia, P., et al. (2013). Learning from nature to improve the heat generation of iron-oxide nanoparticles for magnetic hyperthermia applications. *Sci. Rep.* 3:1652. doi: 10.1038/srep01652

Mello, P. D., Bian, S., Savio, L. E. B., Zhang, H. H., Zhang, J. P., Junger, W., et al. (2017). Hyperthermia and associated changes in membrane fluidity potentiate P2X7 activation to promote tumor cell death. *Oncotarget* 8, 67254–67268. doi: 10.18632/oncotarget.18595

Milowsky, M. I., Galsky, M. D., Morris, M. J., Crona, D. J., George, D. J., Dreicer, R., et al. (2016). Phase 1/2 multiple ascending dose trial of the prostate-specific membrane antigen-targeted antibody drug conjugate MLN2704 in metastatic castration-resistant prostate cancer. *Urol. Oncol.* 34, 530.e515–530.e21. doi: 10.1016/j.urolonc.2016.07.005

Nair, B. G., Nagaoka, Y., Morimoto, H., Yoshida, Y., Maekawa, T., and Kumar, D. S. (2010). Aptamer conjugated magnetic nanoparticles as nanosurgeons. *Nanotechnology* 21:455102. doi: 10.1088/0957-4484/21/45/455102

Namiki, Y., Namiki, T., Yoshida, H., Ishii, Y., Tsubota, A., Koido, S., et al. (2009). A novel magnetic crystal-lipid nanostructure for magnetically guided *in vivo* gene delivery. *Nat. Nanotechnol.* 4, 598–606. doi: 10.1038/nnano.2009.202

Nemati, Z., Alonso, J., Martinez, L. M., Khurshid, H., Garaio, E., Garcia, J. A., et al. (2016). Enhanced magnetic hyperthermia in iron oxide nano-octopods: size and anisotropy effects. *J. Phys. Chem. C* 120, 8370–8379. doi: 10.1021/acs.jpcc.6b01426

Nieskoski, M. D., and Trembly, B. S. (2014). Comparison of a single optimized coil and a helmholtz pair for magnetic nanoparticle hyperthermia. *IEEE T Bio-Med. Eng.* 61, 1642–1650. doi: 10.1109/TBME.2013.2296231

Obaidat, I. M., Issa, B., and Haik, Y. (2015). Magnetic properties of magnetic nanoparticles for efficient hyperthermia. *Nanomaterials* 5, 63–89. doi: 10.3390/nano5010063

Oliveira, T. R., Stauffer, P. R., Lee, C. T., Landon, C. D., Etienne, W., Ashcraft, K. A., et al. (2013). Magnetic fluid hyperthermia for bladder cancer: a preclinical dosimetry study. *Int. J. Hyperthermia* 29, 835–844. doi: 10.3109/02656736.2013.834384

Overgaard, J. (1984). Formula to estimate the thermal enhancement ratio of a single simultaneous hyperthermia and radiation treatment. *Acta Radiol. Oncol.* 23, 135–139. doi: 10.3109/02841868409136001

Overgaard, J., Gonzalez Gonzalez, D., Hulshof, M. C., Arcangeli, G., Dahl, O., Mella, O., et al. (1996). Hyperthermia as an adjuvant to radiation therapy of recurrent or metastatic malignant melanoma. A multicentre randomized trial by the European Society for Hyperthermic Oncology. *Int. J. Hyperthermia* 12, 3–20. doi: 10.3109/02656739609023685

Peer, A. J., Grimm, M. J., Zynda, E. R., and Repasky, E. A. (2010). Diverse immune mechanisms may contribute to the survival benefit seen in cancer patients receiving hyperthermia. *Immunol. Res.* 46, 137–154. doi: 10.1007/s12026-009-8115-8

Peng, H., Tang, J., Zheng, R., Guo, G., Dong, A., Wang, Y., et al. (2017). Nuclear-targeted multifunctional magnetic nanoparticles for photothermal therapy. *Adv. Healthc. Mater.* 6:1601289. doi: 10.1002/adhm.201601289

Perez, C. A., Pajak, T., Emami, B., Hornback, N. B., Tupchong, L., and Rubin, P. (1991). Randomized phase III study comparing irradiation and hyperthermia with irradiation alone in superficial measurable tumors. Final report by the radiation therapy oncology group. *Am. J. Clin. Oncol.* 14, 133–141. doi: 10.1097/00000421-199104000-00008

Phong, P. T., Nam, P. H., Manh, D. H., and Lee, I. J. (2017). $Mn_{0.5}Zn_{0.5}Fe_2O_4$ nanoparticles with high intrinsic loss power for hyperthermia therapy. *J. Magn. Magn. Mater.* 433, 76–83. doi: 10.1016/j.jmmm.2017.03.001

Plan Sangnier, A., Preveral, S., Curcio, A., Silva, A. K. A., Lefèvre, C. T., et al. and Wilhelm, C. (2018). Targeted thermal therapy with genetically engineered magnetite magnetosomes@RGD: Photothermia is far more efficient than magnetic hyperthermia. *J. Control. Release* 279, 271–281. doi: 10.1016/j.jconrel.2018.04.036

Qiu, Y., Tong, S., Zhang, L., Sakurai, Y., Myers, D. R., Hong, L., et al. (2017). Magnetic forces enable controlled drug delivery by disrupting endothelial cell-cell junctions. *Nat. Commun.* 8:15594. doi: 10.1038/ncomms15594

Rau, B., Wust, P., Tilly, W., Gellermann, J., Harder, C., Riess, H., et al. (2000). Preoperative radiochemotherapy in locally advanced or recurrent rectal cancer: regional radiofrequency hyperthermia correlates with clinical parameters. *Int. J. Radiat. Oncol. Biol. Phys.* 48, 381–391. doi: 10.1016/S0360-3016(00)00650-7

Reddy, L. H., Arias, J. L., Nicolas, J., and Couvreur, P. (2012). Magnetic nanoparticles: design and characterization, toxicity and biocompatibility, pharmaceutical and biomedical applications. *Chem. Rev.* 112, 5818–5878. doi: 10.1021/cr300068p

Richter, K., Haslbeck, M., and Buchner, J. (2010). The heat shock response: life on the verge of death. *Mol. Cell* 40, 253–266. doi: 10.1016/j.molcel.2010.10.006

Rivas, J., Banobre-Lopez, M., Pineiro-Redondo, Y., Rivas, B., and Lopez-Quintela, M. A. (2012). Magnetic nanoparticles for application in cancer therapy. *J. Magn. Magn. Mater.* 324, 3499–3502. doi: 10.1016/j.jmmm.2012.02.075

Roizin-Towle, L., and Pirro, J. P. (1991). The response of human and rodent cells to hyperthermia. *Int. J. Radiat. Oncol. Biol. Phys.* 20, 751–756. doi: 10.1016/0360-3016(91)90018-Y

Rosenblum, D., Joshi, N., Tao, W., Karp, J. M., and Peer, D. (2018). Progress and challenges towards targeted delivery of cancer therapeutics. *Nat. Commun.* 9:1410. doi: 10.1038/s41467-018-03705-y

Rosensweig, R. E. (2002). Heating magnetic fluid with alternating magnetic field. *J. Magn. Magn. Mater.* 252, 370–374. doi: 10.1016/S0304-8853(02)00706-0

Ruta, S., Chantrell, R., and Hovorka, O. (2015). Unified model of hyperthermia via hysteresis heating in systems of interacting magnetic nanoparticles. *Sci. Rep.* 5:9090. doi: 10.1038/srep09090

Salas, G., Camarero, J., Cabrera, D., Takacs, H., Varela, M., Ludwig, R., et al. (2014). Modulation of magnetic heating via dipolar magnetic interactions in monodisperse and crystalline iron oxide nanoparticles. *J. Phys. Chem. C* 118, 19985–19994. doi: 10.1021/jp5041234

Schilling, D., Kühnel, A., Konrad, S., Tetzlaff, F., Bayer, C., Yaglom, J., et al. (2015). Sensitizing tumor cells to radiation by targeting the heat shock response. *Cancer Lett.* 360, 294–301. doi: 10.1016/j.canlet.2015.02.033

Shah, A., and Dobrovolskaia, M. A. (2018). Immunological effects of iron oxide nanoparticles and iron-based complex drug formulations: therapeutic benefits, toxicity, mechanistic insights, and translational considerations. *Nanomed. Nanotechnol.* 14, 977–990. doi: 10.1016/j.nano.2018.01.014

Sharma, S., Singhal, S., Sandhu, A. P., Ghoshal, S., Gupta, B. D., and Yadav, N. S. (1991). Local thermo-radiotherapy in carcinoma cervix: improved local control versus increased incidence of distant metastasis. *Asia Oceania J. Obstet. Gynaecol.* 17, 5–12. doi: 10.1111/j.1447-0756.1991.tb00244.x

Sneed, P. K., Stauffer, P. R., Mcdermott, M. W., Diederich, C. J., Lamborn, K. R., Prados, M. D., et al. (1998). Survival benefit of hyperthermia in a prospective randomized trial of brachytherapy boost +/- hyperthermia for glioblastoma multiforme. *Int. J. Radiat. Oncol. Biol. Phys.* 40, 287–295. doi: 10.1016/S0360-3016(97)00731-1

Song, C. W., Park, H. J., Lee, C. K., and Griffin, R. (2005). Implications of increased tumor blood flow and oxygenation caused by mild temperature hyperthermia in tumor treatment. *Int. J. Hyperthermia* 21, 761–767. doi: 10.1080/02656730500204487

Song, C. W., Shakil, A., Osborn, J. L., and Iwata, K. (1996). Tumour oxygenation is increased by hyperthermia at mild temperatures. *Int. J. Hyperthermia* 12, 367–373. doi: 10.3109/02656739609022525

Song, M. J., Zhang, Y., Hu, S. L., Song, L. N., Dong, J. L., Chen, Z. P., et al. (2012). Influence of morphology and surface exchange reaction on magnetic properties of monodisperse magnetite nanoparticles. *Colloid Surface A* 408, 114–121. doi: 10.1016/j.colsurfa.2012.05.039

Sugimachi, K., Kuwano, H., Ide, H., Toge, T., Saku, M., and Oshiumi, Y. (1994). Chemotherapy combined with or without hyperthermia for patients with oesophageal carcinoma: a prospective randomized trial. *Int. J. Hyperthermia* 10, 485–493. doi: 10.3109/02656739409009352

Suriyanto, Ng, E. Y., and Kumar, S.D. (2017). Physical mechanism and modeling of heat generation and transfer in magnetic fluid hyperthermia through Neelian and Brownian relaxation: a review. *Biomed. Eng. Online* 16:36. doi: 10.1186/s12938-017-0327-x

Suto, M., Hirota, Y., Mamiya, H., Fujita, A., Kasuya, R., Tohji, K., et al. (2009). Heat dissipation mechanism of magnetite nanoparticles in magnetic fluid hyperthermia. *J. Magn. Magn. Mater.* 321, 1493–1496. doi: 10.1016/j.jmmm.2009.02.070

Taratula, O., Dani, R. K., Schumann, C., Xu, H., Wang, A., Song, H., et al. (2013). Multifunctional nanomedicine platform for concurrent delivery of chemotherapeutic drugs and mild hyperthermia to ovarian cancer cells. *Int. J. Pharm.* 458, 169–180. doi: 10.1016/j.ijpharm.2013.09.032

Tewes, F., Ehrhardt, C., and Healy, A. M. (2014). Superparamagnetic iron oxide nanoparticles (SPIONs)-loaded Trojan microparticles for targeted aerosol delivery to the lung. *Eur. J. Pharm. Biopharm.* 86, 98–104. doi: 10.1016/j.ejpb.2013.09.004

Thomas, R. G., Moon, M. J., Lee, H., Sasikala, A. R., Kim, C. S., Park, I. K., et al. (2015). Hyaluronic acid conjugated superparamagnetic iron oxide nanoparticle for cancer diagnosis and hyperthermia therapy. *Carbohydr. Polym.* 131, 439–446. doi: 10.1016/j.carbpol.2015.06.010

Toraya-Brown, S., Sheen, M. R., Baird, J. R., Barry, S., Demidenko, E., Turk, M. J., et al. (2013). Phagocytes mediate targeting of iron oxide nanoparticles to tumors for cancer therapy. *Integr. Biol.* 5, 159–171. doi: 10.1039/C2IB20180A

Tse, B. W., Cowin, G. J., Soekmadji, C., Jovanovic, L., Vasireddy, R. S., Ling, M. T., et al. (2015). PSMA-targeting iron oxide magnetic nanoparticles enhance MRI of preclinical prostate cancer. *Nanomedicine* 10, 375–386. doi: 10.2217/nnm.14.122

Urano, M., Kuroda, M., and Nishimura, Y. (1999). For the clinical application of thermochemotherapy given at mild temperatures. *Int. J. Hyperthermia* 15, 79–107. doi: 10.1080/026567399285765

Valdagni, R., Amichetti, M., and Pani, G. (1988). Radical radiation alone versus radical radiation plus microwave hyperthermia for N3 (TNM-UICC) neck nodes: a prospective randomized clinical trial. *Int. J. Radiat. Oncol. Biol. Phys.* 15, 13–24. doi: 10.1016/0360-3016(88)90341-0

van der Zee, J. (2002). Heating the patient: a promising approach? *Ann. Oncol.* 13, 1173–1184. doi: 10.1093/annonc/mdf280

Van Der Zee, J., Gonzalez Gonzalez, D., Van Rhoon, G. C., Van Dijk, J. D., Van Putten, W. L., and Hart, A. A. (2000). Comparison of radiotherapy alone with radiotherapy plus hyperthermia in locally advanced pelvic tumours: a prospective, randomised, multicentre trial. *Dutch. Deep. Hyperthermia Group Lancet* 355, 1119–1125. doi: 10.1016/S0140-6736(00)02059-6

Van Landeghem, F. K., Maier-Hauff, K., Jordan, A., Hoffmann, K. T., Gneveckow, U., Scholz, R., et al. (2009). Post-mortem studies in glioblastoma patients treated with thermotherapy using magnetic nanoparticles. *Biomaterials* 30, 52–57. doi: 10.1016/j.biomaterials.2008.09.044

van Leeuwen, C. M., Oei, A. L., Chin, K. W. T. K., Crezee, J., Bel, A., Westermann, A. M., et al. (2017). A short time interval between radiotherapy and hyperthermia reduces in-field recurrence and mortality in women with advanced cervical cancer. *Radiat. Oncol.* 12:75. doi: 10.1186/s13014-017-0813-0

Vernon, C. C., Hand, J. W., Field, S. B., Machin, D., Whaley, J. B., Van Der Zee, J., et al. (1996). Radiotherapy with or without hyperthermia in the treatment of superficial localized breast cancer: results from five randomized controlled trials. International Collaborative Hyperthermia Group. *Int. J. Radiat. Oncol. Biol. Phys.* 35, 731–744. doi: 10.1016/0360-3016(96)00154-X

Verwaal, V. J., Bruin, S., Boot, H., Van Slooten, G., and Van Tinteren, H. (2008). 8-year follow-up of randomized trial: cytoreduction and hyperthermic intraperitoneal chemotherapy versus systemic chemotherapy in patients with peritoneal carcinomatosis of colorectal cancer. *Ann. Surg. Oncol.* 15, 2426–2432. doi: 10.1245/s10434-008-9966-2

Wei, Y., Zhao, M. Z., Yang, F., Mao, Y., Xie, H., and Zhou, Q. B. (2016). Iron overload by superparamagnetic iron oxide nanoparticles is a high risk factor in cirrhosis by a systems toxicology assessment. *Sci. Rep.* 6:29110. doi: 10.1038/srep29110

Wildeboer, R. R., Southern, P., and Pankhurst, Q. A. (2014). On the reliable measurement of specific absorption rates and intrinsic loss parameters in magnetic hyperthermia materials. *J. Phys. D Appl. Phys.* 47:495003. doi: 10.1088/0022-3727/47/49/495003

Wu, H., Yin, J. J., Wamer, W. G., Zeng, M. Y., and Lo, Y. M. (2014). Reactive oxygen species-related activities of nano-iron metal and nano-iron oxides. *J. Food Drug Anal.* 22, 86–94. doi: 10.1016/j.jfda.2014.01.007

Wust, P., Hildebrandt, B., Sreenivasa, G., Rau, B., Gellermann, J., Riess, H., et al. (2002). Hyperthermia in combined treatment of cancer. *Lancet Oncol.* 3, 487–497. doi: 10.1016/S1470-2045(02)00818-5

Xu, C., Shi, S. X., Feng, L. Z., Chen, F., Graves, S. A., Ehlerding, E. B., et al. (2016). Long circulating reduced graphene oxide-iron oxide nanoparticles for efficient

tumor targeting and multimodality imaging. *Nanoscale* 8, 12683–12692. doi: 10.1039/C5NR09193D

Yang, R., Tang, Q. S., Miao, F. Q., An, Y. L., Li, M. F., Han, Y., et al. (2015). Inhibition of heat-shock protein 90 sensitizes liver cancer stem-like cells to magnetic hyperthermia and enhances anti-tumor effect on hepatocellular carcinoma-burdened nude mice. *Int. J. Nanomedicine* 10, 7345–7358. doi: 10.2147/IJN.S93758

Zhang, J., Dewilde, A. H., Chinn, P., Foreman, A., Barry, S., Kanne, D., et al. (2011). Herceptin-directed nanoparticles activated by an alternating magnetic field selectively kill HER-2 positive human breast cells *in vitro* via hyperthermia. *Int. J. Hyperthermia* 27, 682–697. doi: 10.3109/02656736.2011.609863

Zhu, W. G., Antoku, S., Kura, S., Aramaki, R., Nakamura, K., and Sasaki, H. (1995). Enhancement of hyperthermic killing in L5178y cells by protease inhibitors. *Cancer Res.* 55, 739–742.

Zuckerman, J. E., and Davis, M. E. (2015). Clinical experiences with systemically administered siRNA-based therapeutics in cancer. *Nat. Rev. Drug Discov.* 14, 843–856. doi: 10.1038/nrd4685

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MINI REVIEW
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Nano-BCG: A Promising Delivery System for Treatment of Human Bladder Cancer

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Mycobacterium bovis bacillus Calmette–Guerin (BCG) remains at the forefront of immunotherapy for treating bladder cancer patients. However, the incidence of recurrence and progression to invasive cancer is commonly observed. There are no established effective intravesical therapies available for patients, whose tumors recur following BCG treatment, representing an important unmet clinical need. In addition, there are very limited options for patients who do not respond to or tolerate chemotherapy due to toxicities, resulting in poor overall treatment outcomes. Within this context, nanotechnology is an emergent and promising tool for: (1) controlling drug release for extended time frames, (2) combination therapies due to the ability to encapsulate multiple drugs simultaneously, (3) reducing systemic side effects, (4) increasing bioavailability, (5) and increasing the viability of various routes of administration. Moreover, bladder cancer is often characterized by high mutation rates and over expression of tumor antigens on the tumor cell surface. Therapeutic targeting of these biomolecules may be improved by nanotechnology strategies. In this mini-review, we discuss how nanotechnology can help overcome current obstacles in bladder cancer treatment, and how nanotechnology can facilitate combination chemotherapeutic and BCG immunotherapies for the treatment of non-muscle invasive urothelial bladder cancer.

Keywords: bladder cancer, nanotechnology, BCG, Nano-BCG, monoclonal antibody, EGFR

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INTRODUCTION

Bladder cancer (BC) is the second most common malignancy of the urinary tract, the fourth most common cancer in men with a yearly incidence rate of 330, 380 cases, and the 11th most common among women with a yearly incidence rate of 99,413. Worldwide, carcinomas of the bladder represent the ninth most common cause of cancer, with 430,000 patients diagnosed with BC annually (Jemal et al., 1999; Ferlay et al., 2013). The incidence of BC also increases with advancing age, as 90% of new diagnoses are made in people over the age of 55 (average age of 73 years at diagnosis) (American Cancer Society, 2017).

Most (75%) BC cases are non-muscle-invasive bladder cancers (NMIBC) at diagnosis with the other 25% representing muscle invasive bladder cancers (MIBC) or metastatic cancers (Moch et al., 2016). Urothelial carcinomas can be categorized as low grade or high grade according to their

architectural and cytological atypia and include papillary urothelial neoplasm or low malignant potential (Cheng et al., 2012). Pathological assessment is the gold standing for tumor classification. Ta (non-invasive papillary) and Tis [carcinoma *in situ* (CIS)] are tumors that are restricted to the mucosa, while T1 and T2 are tumors that invade the lamina propria and the muscularis propria, respectively (Sobin and Gospodarowicz, 2009).

Initial BC treatments involve transurethral resection (TURBT) to facilitate removal of the visible tumor (Hall et al., 2007; Sylvester, 2008; Babjuk et al., 2013). Further therapy is dependent on pathologic stage and grade of the tumor and often mediated through intravesical instillation. Although the response rate to therapy in patients with NMIBC is high (∼80%), 50–90% of NMIBC patients suffer from recurrence within 5 years, with muscle invasion found in up to 20% of recurrent patients (Rübben et al., 1988; Lamm and Allaway, 2000; Hussain et al., 2009). This review focuses on currently available BC therapies and describes nanotechnology tools to enhance therapeutic effects and overcome side effects, emphasizing its use to improve BCG immunotherapy.

BLADDER CANCER TREATMENTS

Following TURBT, a single intravesical chemotherapy treatment is recommended for patients with low to intermediate risk NMIBC, with mitomycin, epirubicin, and gemcitabine representing common drugs of choice (Kamat et al., 2016). It has been shown that the relative risk for tumor recurrence is reduced by 50% if the chemotherapy instillation is given with 24 h after TURBT (Kaasinen et al., 2002).

Intravesical immunotherapy with *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is the treatment of choice for patients with high-risk NMIBC. BCG immunotherapy is the gold standard treatment for NMIBC due to its ability to reduce recurrence and progression to MIBC (Ahn et al., 2014). A meta-analysis with individual patient data comparing BCG immunotherapy with intravesical mitomycin chemotherapy has shown BCG to be superior in terms of reducing recurrence and delaying disease progression; however, no significant differences in progression or overall survival were observed (Malmström et al., 2009). Conventional BCG treatment consists of a percutaneous BCG vaccine administered 2–6 weeks after TURBT followed by 6 weekly courses of intravesical BCG administration (Morales et al., 1976; Kresowik, 2009; Kamat et al., 2015). However, specific BCG substrain preferences, schedules, and dosages differ across geographic regions due the wide range of BCG substrains licensed for human tuberculosis vaccination and BCG immunotherapy (Gan et al., 2013). Based on multiple meta-analyses, it is recommended to continue BCG therapy for 1 to 3 years if tolerated by the patient to decrease recurrence and progression of NMIBC (Shelley et al., 2001; Sylvester et al., 2002; Böhle and Bock, 2004; Hall et al., 2007; Gontero et al., 2010; Babjuk et al., 2013).

Despite these guidelines, it is estimated that 20% of patients with high-risk NMIBC treated with BCG will progress to muscle invasion or suffer from NMIBC recurrence within 5 years (Rübben et al., 1988). MIBC is a major clinical issue due to its aggressiveness and high 5 year mortality rate. To maximize survival rates, radical cystectomy (RC) represents the best treatment option. RC consists of removal of the bladder, prostate, seminal vesicles, proximal vas deferens, and proximal urethra in men, and bladder, uterus, ovaries, fallopian tubes, urethra, and part of vagina in women (Arcangeli et al., 2015). Many patients cannot tolerate the morbidity of RC and instead opt for continued local therapy as an effort to spare their bladder (Ahn et al., 2014). Some studies have shown progress with the use of Mytomicin C (van der Heijden et al., 2004; Halachmi et al., 2011), Gemcitabine (Skinner et al., 2013), Valrubicin (Steinberg et al., 2000), Docetaxel (Barlow et al., 2013), Nab-Paclitaxel (McKiernan et al., 2011), mycobacterial cell wall extract (Morales et al., 2009), EGFR (Rebouissou et al., 2014) and a combination therapy of Gemcitabine and Mytomicin C for the treatment of BC (Lightfoot et al., 2011). More recently, trimodal treatment with simultaneous delivery of chemotherapy and radiotherapy has emerged as an effective bladder sparing treatment with similar survival rates compared to RC (Arcangeli et al., 2015). However, none of these local therapies have shown to be more effective for BC control than early RC (Ahn et al., 2014).

CHEMOTHERAPY OPTIMIZATION USING NANOTECHNOLOGY FOR BLADDER CANCER THERAPY

Nanotechnology consists in the study and application of materials on the nanometer scale (Ebbesen and Jensen, 2006) and application of nanotechnology in the medical field is referred to as nanomedicine (Sweeney, 2015). Nanotechnology have proven to be a powerful tool for the development of new chemotherapies or immunotherapies for BC. The development of new drug delivery systems has been growing and is expected to continue to increase over the next few years (Brito et al., 2017).

In this context, several studies have utilized nanoparticles (NPs) to increase the therapeutic effectiveness and reduce adverse effects of chemotherapy by targeting chemotherapeutic agents to a specific tissue and increasing its bioavailability (Yurgel et al., 2014; Kang et al., 2017; Yao et al., 2017; Zhu et al., 2017). Polysaccharide-based NPs loaded with Mitomycin C and surrounded by the bioadhesive polymer chitosan mixed with polylactic acid or with poly(ε-caprolactone) have been utilized in an attempt to optimize BC treatments. This NP promoted favorable drug loading and release profiles along with improved anticancer efficacy and cellular interactions (Bilensoy et al., 2009). Erdogar et al. (2012) has also demonstrated that bioadhesive and cationic NPs loaded with Mitomycin C are able to increased exposure of the bladder to the drug resulting in a drug reservoir at the action site, which might improve local treatment (Erdogar et al., 2012). In addition, cationic core-shell nanoparticles loaded with Mitomycin C have also improved antitumor efficacy in tumor-induced rat models (Erdogar et al., 2014).

Magnetic NPs (MNPs) also show promise for delivery of quimiotherapic agents to the target tissue (Stapf et al., 2017). To limit doxorubicin's (Dox) cytotoxic effects on healthy cells, MNPs (iron oxide) were conjugated with Dox to ensure efficient delivery

to cancer sites, resulting in increased BC sensitivity compared to Dox alone (Nowicka et al., 2013). In addition, monoclonal antibodies (mABs) bound to MNPs can increase the ability of MNPs to target BC cells and enable thermotherapy to cope with BC recurrence (Rezaei et al., 2017).

NPs loaded with molecules with high urothelium mucoadhesivity is another approach used to optimize the delivery of molecules to the target tissue. To increase Dox specificity for BC cells, thiol-functionalized NPs loaded with Dox were synthesized and induced cytotoxicity against UMUC3 cancer cells (Zhang et al., 2014). Besides the possibility of conjugation with different molecules, studies have also reported the importance of developing methods to modify the surface of mesoporous silica NPs to enhance the antineoplasic effects of Dox on BC (Wei et al., 2017). An enhanced therapeutic effect against UMUC3 cells was also demonstrated using Dox and peptide-modified cisplatin synergistically loaded onto positively charged mucoadhesive chitosan–polymethacrylic acid nanocapsules (Lu et al., 2016).

In addition, platinum agents can be loaded onto a variety of polymeric, lipid, and inorganic nanocarriers, including liposomes, NPs, and nanotubes to increase their antitumoral effects (Browning et al., 2017). The use of cisplatin nanocarriers is associated with reduced toxicity and adverse events (Sudha et al., 2017); however, novel strategies are required to increase drug uptake and release at the target site. In this regard, cisplatin NPs were evaluated in a preclinical study against NMIBC and cisplatin-loaded biocompatible poly(L-aspartic acid sodium salt) (PAA) NPs demonstrated potential for improved intravesical treatment of NMIBC while reducing local and systemic side effect (Kates et al., 2017).

Nanotechnology tools have also been used in clinical trials. Albumin-bound-Paclitaxel NPs showed minimal toxicity and systemic absorption when used to treat NMIBC during the first human intravesical phase I trial (McKiernan et al., 2011). In addition, phase II trials have demonstrated minimal toxicity of intravesical nab-paclitaxel in NMIBC patients with a response rate of 35.7% (Mckiernan et al., 2014). The formulation of albumin-bound-paclitaxel NPs has also been used for the first time to treat unresectable metastatic urethral cancer. Follwoing therapy, a 70% reduction in the size of the tumor was observed in addition to 19 months of progression free survival (Abaza and Alemany, 2014).

RNAI OPTIMIZATION OF NANOTECHNOLOGY BLADDER CANCER THERAPY

The use of interference RNA (RNAi) combined with nanotechnology is another promising approach for BC treatment. RNAi technology can be used to inhibit tumor growth through messenger RNA inhibition of several activated oncogenes (Xin et al., 2017). In human BC, some upregulated genes associated to the development of resistance to chemotherapy have been inhibited using RNAi technology through knockdown of the target gene (Pan et al., 2016; Wang et al., 2017). Within this context, RNAi technology is a highly effective approach to combat chemoresistance and improve advanced BC outcomes.

Although RNAi technology could be used to overcome multidrug resistance and restore cells sensitivity, there are several challenges associated with RNAi delivery to diseased sites for gene therapy (Melamed et al., 2017). NPs appear to be a promising tool to help overcome existing biological barriers to RNAi delivery. Through this approach, studies have reported upregulation of BC specific genes, which can be effective targets of NP-siRNA therapeutic approaches (Seth et al., 2011; Müller et al., 2016).

Small dsRNAs, known as small activating RNA (saRNA), produce the opposite effect of RNAi by inducing gene expression (Li et al., 2006; Chen et al., 2008). The therapeutic potential of dsRNA (P21-322) coupled with nanotechnology has been demonstrated using a 20-fluoro-modified derivative loaded into lipid NPs in an orthotopic model of BC. Antitumoral activity and induction of p21 expression was confirmed *in vitro* and *in vivo* (Kang et al., 2012), suggesting induction of specific genes can provide an alternative route to BC treatment.

FUNCTIONALIZATION OF NANOPARTICLES TO OPTIMIZE BLADDER CANCER THERAPY

The functionalization of nanoparticles with monoclonal antibodies is a promising strategy for targeted delivery to and uptake by cells overexpressing the antigens specific for these antibodies (Eloy et al., 2017). Development of molecules that exhibit affinity for targets expressed on tumor cells represents an emerging therapeutic approach (Diesendruck and Benhar, 2017). For example, the receptor tyrosine kinase EGFR exhibits altered expression in several types of solid tumors and its overexpression in UC is directly correlated with advanced tumor stages (Kassouf et al., 2008). The incidence of EGFR positivity in epithelial tumors varies by tumor type. BC is often characterized by a high mutation rate and high EGFR expression in approximately 50% of cases (Colquhoun and Mellon, 2002). Thanks to the expression of EGRF on BC cells, an anti-EGFR monoclonal antibody conjugated to gold nanorods was able to effectively bind EGFR-expressing BC cells and reduce the systemic exposure and clearance of nanoparticles from the body (Yang et al., 2015). These results indicate the use of monoclonal antibodies represents another possible approach for delivery of molecules to specific tissues.

NANO-BCG: OPTIMIZING BCG IMMUNOTHERAPY USING NANOTECHNOLOGY TO TREAT BLADDER CANCER

As previously mentioned, BCG is considered the standard treatment for NMIBC. However, BCG immunotherapy

TABLE 1 | Major studies involving nanotechnology tools applied to BCG immunotherapy against bladder cancer.

Study objective	Approach used	Nanoparticle name	Preparation method	Mean nanoparticle size	Zeta potential (mv)	Entrapment efficiency	Main results	Model	Reference
To develop a magnetic thermosensitive hydrogel for intravesical Bacillus Calmette-Guérin (BCG) delivery formulated with chitosan (CS), b-glycerophosphate (GP), and Fe_3O_4 magnetic nanoparticles (Fe_3O_4-MNP).	Magnetic chitosan hydrogel	Fe_3O_4-MNP	Solvent evaporation	186.2 nm	Positive: 38.4	89.73%	†A magnetic thermosensitive CS/GP hydrogel was a suitable matrix for extended BCG delivery by intravesical route. †The biodegradable and injectable thermosensitive gel showed a rapid solegel phase transformation. †Sustained delivery of BCG increased the antitumor efficacy and induced a high local immunity in bladder.	*In vivo* (Female Wistar rats-8-weeks old)	Zhang et al., 2013
To optimize and evaluate the antitumor efficacy of cationic chitosan (CS) nanoparticles encapsulating BCG for bladder tumor	Cationic chitosan (CS) nanoparticles encapsulating BCG	BCG-loaded CS	Data not shown	269–375 nm	Positive	42%	†CG-loaded chitosan nanoparticles resulted in increased survival rate. †Significant nanoparticle accumulation in bladder tissues was observed. †Cationic CS nanoparticles provide a significantly improved intravesical immunotherapy approach for bladder tumors.	*In vivo* (Rats)	Erdogar et al., 2014
To determine the direct effect of viable or heat-killed BCG and BCG cell wall skeleton (BCG-CWS) on UC cells *In vitro*	BCG Cell Wall Skeleton (BCG-CWS)	SMP105 BCG-CWS	Preparation of cell wall skeleton (CWS) (SMP-105) according Azuma et al. (1974) and Uenishi et al. (2007)	4.7 to 67.8 μm	Data not shown	Data not shown	†BCG induced cell growth retardation in highly malignant UC expressing integrin α5β1 (VLA5), suggesting VLA5 may be a biomarker of UC with sensitivity to BCG. †BCG-CWS is a promising substance which might replace BCG, preventing complications of viable BCG treatment.	*In vitro* (bladder cancer cell lines T24, HT1376, and RT4)	Kato et al., 2010
To evaluate of the ability of natural killer cells to cytolyze bladder cancer cells modified by R8-liposome-bacillus Calmette-Guérin (BCG)-cell wall skeleton (CWS) treatment	BCG Cell Wall Skeleton (BCG-CWS)	R8-liposome-bacillus Calmette-Guéin (BCG)-cell wall skeleton (CWS)	R8-liposome-BCG-CWS was prepared using a method described previously Homhuan et al. (2007)	Data not shown	Data not shown	Data not shown	†The induction of surface NKG2D ligands by R8-liposomeBCG-CWS rendered cancer cells more susceptible to cytolysis by lymphokine-activated killing. †T24 cells and RT-112 cells can directly respond to R8-liposome-BCG-CWS.	*In vitro* (T24 cells and RT-112 cells)	Miyazaki et al., 2011a

(Continued)

TABLE 1 | Continued

Study objective	Approach used	Nanoparticle name	Preparation method	Mean nanoparticle size	Zeta potential (mv)	Entrapment efficiency	Main results	Model	Reference
To determine if a non-living bacterial agent could be as efficacious as live BCG in a model of bladder cancer	BCG Cell Wall Skeleton (BCG-CWS)	R8-liposome-BCG-CWS	R8-liposome-BCG-CWS was prepared using a method described previously Homhuan et al. (2007)	Data not shown	Data not shown	Data not shown	†Rats receiving R8-liposome-BCG-CWS intravesically developed significantly fewer tumors. †R8-liposomeBCG-CWS significantly inhibited rat bladder carcinogenesis.	*In vivo* (8-week-old male Fisher-344 rats)	Miyazaki et al., 2011b
To develop a cell wall (CW) preparation from heat-killed bacillus Calmette-Guérin (BCG-CW) incorporated into octaarginine-modified cationized liposomes and to evaluate its immunoprotective potentiation in mice.	BCG Cell Wall Skeleton (BCG-CWS)	R8-liposome-BCG-CW	The CW fraction was prepared from heat-killed *Mycobacterium bovis* BCG Tokyo 172 cells according Joraku et al., 2008	232 to 270 nm	Positive: 19.9 to 26.9	Data not shown	†Confocal laser scanning microscopy showed enhanced incorporation of R8-liposome-BCG-CW into MBT-2 cells after 1 h of co-incubation. †0.1 mg R8-liposome-BCG-CW completely inhibited the growth of MBT-2 tumors while 0.1mg BCG-CW alone did not.	*In vitro* (bladder cancer cell line - MBT-2) and *In vivo* (female C3H/HeN mice - 7-weeks-old)	Joraku et al., 2008
To investigate the role of bladder cancer cells and DCs in internalization of BCG-CWS and initiation of the antibladder tumor effect using CWS-NP	Nanoparticle encapsulating BCG-CWS	CWS-NP	CWS-NP was prepared by the LEEL method described by Nakamura et al., 2014a	173 ± 8 nm	Positive: 41 ± 1	Data not shown	†Immune responses caused by the internalization of BCG-CWS by bladder cancer cells. †Tumor growth was significantly inhibited in mice that had been inoculated with mouse bladder cancer (MBT-2) cells containing internalized BCG-CWS.	*In vitro* (bladder cancer cell line - MBT-2) and *In vivo* (C3H/HeN mice (female, 8–10 weeks)	Nakamura et al., 2014a
To develop a novel packaging method that permits BCG-CWS to be encapsulated into lipid particles, as well as evaluate uptake efficiency of CWS-NP by mouse bladder tumor (MBT-2) cells *In vitro* and resulting tumor growth inhibition in mice bearing MBT-2 tumors	BCG-CWS encapsulated into lipid particles	CWS-NP	Preparation of CWS-NP by the LEEL method and the hydration methodh by Nakamura et al., 2014a	The diameter and zeta-potential of R8-Lip were 283 ± 16 nm and the diameter of CWS-NP/LEEL were 166 ± 2 nm.	The zeta-potential of R8-lip were 48 ± 2 and the zeta-potential of CWS-NP/LEEL 31 ± 0.4	The encapsulating ratio of BCG-CWS in the CWS-NP/LEEL was 57 ± 2%.	†CWS-NP was efficiently taken up by mouse bladder tumor (MBT-2) cells *In vitro* and inhibited tumor growth in mice bearing MBT-2 tumors. †Intravesically administered CWS-NP showed significant antitumor effects in a rat model presenting with naturally developed bladder tumors.	*In vitro (Bladder cancer cells - MBT-2) and In vivo* (Female C3H/HeN mice– 7 weeks old)	Nakamura et al., 2014b

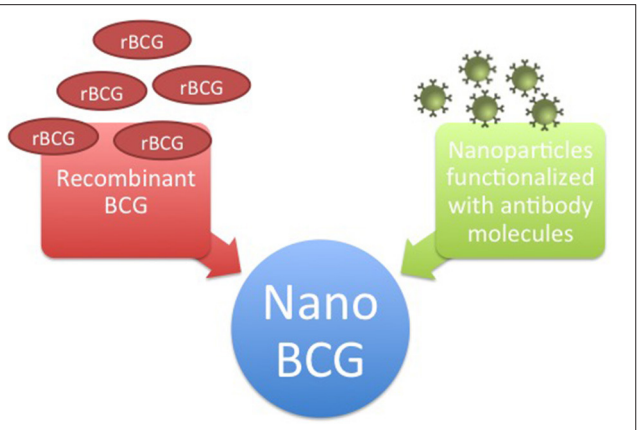

FIGURE 1 | Combination of therapeutic approaches for bladder cancer treatment. Nano BCG has gain of effect by combination with recombinant BCG or by functionalization of nanoparticles with antibodies molecules.

is associated with frequent induction of adverse effects in patients (Poletajew et al., 2017) leading researchers to investigate novel alternatives to increase their effectiveness (Begnini et al., 2013). Delivery systems and nanotechnological approaches are interesting tools to improve currently available BCG therapies and prolong exposure of the bladder tissue. The main advances of nanotechnology tools for improvement of BCG immunotherapies against BC are listed in **Table 1**.

Cationic Chitosan (CS) NPs encapsulating BCG developed with the purpose of increasing the antitumor efficacy of BCG following intravesical administration have demonstrated significant advantages for the treatment of BC (Erdogar et al., 2014). Application of magnetic thermosensitive hydrogel developed with chitosan has been shown to be effective in increasing exposure of the bladder to BCG in addition to potentializing its immunological response (Zhang et al., 2013).

With the goal of overcoming side effects resulting from the administration of viable BCG, BCG cell wall skeleton (BCG-CWS) used as an immunomodulator in cancer patients (Uenishi et al., 2007, 2009; Hayashi et al., 2009) was shown to effectively control cell proliferation in UC, representing an efficient and safe alternative to BC immunotherapy (Kato et al., 2010). Nakamura et al. (2014a) was the first to develop a nanoencapsulated BCG-CWS (CWS-NP) using a liposome evaporated emulsified lipid (LEEL) method, resulting in a strong antitumor effect against MBT-2 BC cells as well as *in vivo* induced tumors (Nakamura et al., 2014a). CWS-NP was also shown to produce significant antitumor effects through internalization of BCG-CWS in BC cells, which contributes to the initiation of antitumor immunological activity (Nakamura et al., 2014b).

Another nanotechnological approach applied to BCG therapy consists of the use of modified nanoparticles incorporating BCG cell wall (BCG-CW) or skeleton. Utilization of octaarginine-modified liposomes incorporating BCG-CW (R8-liposome-BCG-CW) results in increased immunotherapeutic potential of BCG-CW for NMIBC through cellular internalization resulting in growth inhibition *in vivo* (Joraku et al., 2008). R8-liposome-BCG-CWS has also be used to investigate the suppressive effects of liposomes using a rat N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) induced BC model. This approach demonstrated that R8-liposome-BCG-CWS displays inhibitory effects against CD *in vivo* (Miyazaki et al., 2011b). Using this same approach, other studies have demonstrated that R8-liposome-BCG-CWS treatment results in induction of surface specific ligands (NKG2D) in BC cells, making them more susceptible to lymphokine-activated killing (LAK), indicating these cells are affected by R8-liposome BCG-CWS administration (Miyazaki et al., 2011a). These results demonstrate the efficiency of nanotechnology applications for optimization and development of novel BCG immunotherapy approaches for BC.

PERSPECTIVE

Considering the promising results demonstrated by utilizing nanotechnology to develop new BC therapies, including success application of those approaches to BCG therapy, we believe that nanotechnology will provide significant advances for improving BC treatment. In this review, we described the main advances and applications of nanotechnology tools for development of novel treatments against BC, providing evidence that nanotechnology has contributed greatly to this effort by controlling drug release for longer periods, enabling the encapsulation of multiple drugs simultaneously, decreasing side effects, and increasing bioavailability. Nevertheless, we believe that combination therapies show more promise for effective treatment of this complex disease than individual approaches (**Figure 1**).

Although nanotechnology has resulted in significant progress for BCG immunotherapy, including increasing immunotherapeutic effects, prolonging exposure of the bladder tissue, and reducing adverse side effects, we believe this approach can be further enhanced through intravesical BCG delivery using NPs functionalized with antibody molecules against highly expressed receptors on the surface of BC cells such as EGFR to further target drug delivery and avoid systemic exposure and clearance of NPs from the body. Therefore, considering the emerging and motivating results using these approaches to treat BC, we believe that BCG delivery using NPs functionalized with monoclonal antibodies, in particular anti-EGFR, will provide a significantly improved delivery system for treatment of human BC.

AUTHOR CONTRIBUTIONS

JB drafted the manuscript. CB wrote the introduction section, KB wrote the bladder cancer treatments section, and all authors, mainly AP, SG, TC, FS, and KB were responsible for conception, manuscript review, and critical intellectual input.

REFERENCES

Abaza, Y. M., and Alemany, C. (2014). Nanoparticle albumin-bound-paclitaxel in the treatment of metastatic urethral adenocarcinoma: the significance of molecular profiling and targeted therapy. *Case Rep. Urol.* 2014, 1–3. doi: 10.1155/2014/489686

Ahn, J. J., Ghandour, R. A., and McKiernan, J. M. (2014). New agents for bacillus Calmette-Guérin-refractory nonmuscle invasive bladder cancer. *Curr. Opin. Urol.* 24, 540–545. doi: 10.1097/MOU.0000000000000088

Arcangeli, G., Strigari, L., and Arcangeli, S. (2015). Radical cystectomy versus organ-sparing trimodality treatment in muscle-invasive bladder cancer: a systematic review of clinical trials. *Crit. Rev. Oncol. Hematol.* 95, 387–396. doi: 10.1016/j.critrevonc.2015.04.006

Azuma, I., Ribi, E. E., Meyer, T. J., and Zbar, B. (1974). Biologically active components from mycobacterial cell walls. I. Isolation and composition of cell wall skeleton and component P3. *J. Natl. Cancer Inst.* 52, 95–101. doi: 10.1093/jnci/52.1.95

Babjuk, M., Burger, M., Zigeuner, R., Shariat, S. F., van Rhijn, B. W. G., Compérat, E., et al. (2013). EAU guidelines on non-muscle-invasive Urothelial carcinoma of the bladder: update 2013. *Eur. Urol.* 64, 639–653. doi: 10.1016/j.eururo.2013.06.003

Barlow, L. J., McKiernan, J. M., and Benson, M. C. (2013). Long-term survival outcomes with intravesical docetaxel for recurrent nonmuscle invasive bladder cancer after previous bacillus Calmette-Guérin therapy. *J. Urol.* 189, 834–839. doi: 10.1016/j.juro.2012.10.068

Begnini, K. R., Rizzi, C., Campos, V. F., Borsuk, S., Schultze, E., Yurgel, V. C., et al. (2013). Auxotrophic recombinant *Mycobacterium bovis* BCG overexpressing Ag85B enhances cytotoxicity on superficial bladder cancer cells *in vitro*. *Appl. Microbiol. Biotechnol.* 97, 1543–1552. doi: 10.1007/s00253-012-4416-2

Bilensoy, E., Sarisozen, C., Esendagli, G., Dogan, A. L., Aktaş, Y., Sen, M., et al. (2009). Intravesical cationic nanoparticles of chitosan and polycaprolactone for the delivery of Mitomycin C to bladder tumors. *Int. J. Pharm.* 371, 170–176. doi: 10.1016/j.ijpharm.2008.12.015

Böhle, A., and Bock, P. R. (2004). Intravesical bacille Calmette-Guérin versus mitomycin C in superficial bladder cancer: formal meta-analysis of comparative studies on tumor progression. *Urology* 63, 682–686; discussion 686–687. doi: 10.1016/j.urology.2003.11.049

Brito, O. M., Mônica, V. N., and Luciano, B. M. (2017). A review of recent developments on micro/nanostructured pharmaceutical systems for intravesical therapy of the bladder cancer. *Pharm. Dev. Technol.* 12, 1–15. doi: 10.1080/10837450.2017.1312441

Browning, R. J., Reardon, P. J. T., Parhizkar, M., Pedley, R. B., Edirisinghe, M., Knowles, J. C., et al. (2017). Drug delivery strategies for platinum based chemotherapy. *ACS Nano* 11, 8560–8578. doi: 10.1021/acsnano.7b04092

Chen, Z., Place, R. F., Jia, Z.-J., Pookot, D., Dahiya, R., and Li, L.-C. (2008). Antitumor effect of dsRNA-induced p21WAF1/CIP1 gene activation in human bladder cancer cells. *Mol. Cancer Ther.* 7, 698–703. doi: 10.1158/1535-7163.MCT-07-2312

Cheng, L., MacLennan, G. T., and Lopez-Beltran, A. (2012). Histologic grading of urothelial carcinoma: A reappraisal. *Hum. Pathol.* 43, 2097–2108. doi: 10.1016/j.humpath.2012.01.008

Colquhoun, A. J., and Mellon, J. K. (2002). Epidermal growth factor receptor and bladder cancer: a review. *Urol. Int.* 48, 365–371. doi: 10.1136/pmj.78.924.584

Diesendruck, Y., and Benhar, I. (2017). Novel immune check point inhibiting antibodies in cancer therapy-opportunities and challenges. *Drug Resist. Updat.* 30, 39–47. doi: 10.1016/j.drup.2017.02.001

Ebbesen, M., and Jensen, T. G. (2006). Nanomedicine: techniques, potentials, and ethical implications. *J. Biomed. Biotechnol.* 2006:51516. doi: 10.1155/JBB/2006/51516

Eloy, J. O., Petrilli, R., Trevizan, L. N. F., and Chorilli, M. (2017). Immunoliposomes: a review on functionalization strategies and targets for drug delivery. *Colloids Surf. B Biointerfaces* 159, 454–467. doi: 10.1016/j.colsurfb.2017.07.085

Erdogar, N., Iskit, A. B., Eroglu, H., Sargon, M. F., Mungan, N. A., and Bilensoy, E. (2014). Cationic core-shell nanoparticles for intravesical chemotherapy in tumor-induced rat model: Safety and efficacy. *Int. J. Pharm.* 471, 1–9. doi: 10.1016/j.ijpharm.2014.05.014

Erdogar, N., Iskit, A. B., Mungan, N. A., and Bilensoy, E. (2012). Prolonged retention and *in vivo* evaluation of cationic nanoparticles loaded with Mitomycin C designed for intravesical chemotherapy of bladder tumours. *J. Microencapsul.* 29, 576–582. doi: 10.3109/02652048.2012.668957

Ferlay, J., Steliarova-Foucher, E., Lortet-Tieulent, J., Rosso, S., Coebergh, J. W. W., Comber, H., et al. (2013). Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur. J. Cancer* 49, 1374–1403. doi: 10.1016/j.ejca.2012.12.027

Gan, C., Mostafid, H., Khan, M. S., and Lewis, D. J. M. (2013). BCG immunotherapy for bladder cancer—the effects of substrain differences. *Nat. Rev. Urol.* 10, 580–588. doi: 10.1038/nrurol.2013.194

Gontero, P., Bohle, A., Malmstrom, P. U., O'Donnell, M. A., Oderda, M., Sylvester, R., et al. (2010). The role of bacillus calmette-guérin in the treatment of non-muscle-invasive bladder cancer. *Eur. Urol.* 57, 410–429. doi: 10.1016/j.eururo.2009.11.023

Halachmi, S., Moskovitz, B., Maffezzini, M., Conti, G., Verweij, F., Kedar, D., et al. (2011). Intravesical mitomycin C combined with hyperthermia for patients with T1G3 transitional cell carcinoma of the bladder. *Urol. Oncol. Semin. Orig. Investig.* 29, 259–264. doi: 10.1016/j.urolonc.2009.02.012

Hall, M. C., Chang, S. S., Dalbagni, G., Pruthi, R. S., Seigne, J. D., Skinner, E. C., et al. (2007). Guideline for the management of nonmuscle invasive bladder cancer (stages Ta, T1, and Tis): 2007 update. *J. Urol.* 178, 2314–2330. doi: 10.1016/j.juro.2007.09.003

Hayashi, A., Nishida, Y., Yoshii, S., Kim, S. Y., Uda, H., and Hamasaki, T. (2009). Immunotherapy of ovarian cancer with cell wall skeleton of *Mycobacterium bovis* Bacillus Calmette-Guérin: effect of lymphadenectomy. *Cancer Sci.* 100, 1991–1995. doi: 10.1111/j.1349-7006.2009.01271.x

Homhuan, A., Kogure, K., Akaza, H., Futaki, S., Naka, T., and Fujita, Y. (2007). New packaging method of mycobacterial cell wall using octaarginine-modified liposomes: enhanced uptake by and immunostimulatory activity of dendritic cells. *J. Control. Release* 120, 60–69. doi: 10.1016/j.jconrel.2007.03.017

Hussain, M. H., Wood, D. P., Bajorin, D. F., Bochner, B. H., Dreicer, R., Lamm, D. L., et al. (2009). Bladder cancer: narrowing the gap between evidence and practice. *J. Clin. Oncol.* 27, 5680–5684. doi: 10.1200/JCO.2009.23.6901

Jemal, A., Bray, F., and Ferlay, J. (1999). Global Cancer Statistics: 2011. *CA Cancer J. Clin.* 49, 33–64. doi: 10.3322/canjclin.49.1.33

Joraku, A., Homhuan, A., Kawai, K., Yamamoto, T., Miyazaki, J., Kogure, K., et al. (2008). Immunoprotection against murine bladder carcinoma by octaarginine-modified liposomes incorporating cell wall of *Mycobacterium bovis* bacillus Calmette-Guérin. *BJU Int.* 103, 686–693. doi: 10.1111/j.1464-410X.2008.08235.x

Kaasinen, E., Rintala, E., Hellström, P., Viitanen, J., Juusela, H., Rajala, P., et al. (2002). Factors explaining recurrence in patients undergoing chemoimmunotherapy regimens for frequently recurring superficial bladder carcinoma. *Eur. Urol.* 42, 167–174. doi: 10.1016/S0302-2838(02)00260-9

Kamat, A. M., Flaig, T. W., Grossman, H. B., Konety, B., Lamm, D., O'Donnell, M. A., et al. (2015). Expert consensus document: Consensus statement on best practice management regarding the use of intravesical immunotherapy with BCG for bladder cancer. *Nat. Rev. Urol.* 12, 225–235. doi: 10.1038/nrurol.2015.58

Kamat, A. M., Hahn, N. M., Efstathiou, J. A., Lerner, S. P., Malmström, P. U., Choi, W., et al. (2016). Bladder cancer. *Lancet* 388, 2796–2810. doi: 10.1016/S0140-6736(16)30512-8

Kang, M. R., Yang, G., Place, R. F., Charisse, K., Epstein-Barash, H., Manoharan, M., et al. (2012). Intravesical delivery of small activating RNA formulated into lipid nanoparticles inhibits orthotopic bladder tumor growth. *Cancer Res.* 72, 5069–5079. doi: 10.1158/0008-5472.CAN-12-1871

Kang, S. H., Revuri, V., Lee, S.-J., Cho, S., Park, I.-K., Cho, K. J., et al. (2017). Oral siRNA delivery to treat colorectal liver metastases. *ACS Nano* 11, 10417–10429. doi: 10.1021/acsnano.7b05547

Kassouf, W., Black, P. C., Tuziak, T., Bondaruk, J., Lee, S., Brown, G. A., et al. (2008). Distinctive expression pattern of erbb family receptors signifies an aggressive variant of bladder cancer. *J. Urol.* 179, 353–358. doi: 10.1016/j.juro.2007.08.087

Kates, M., Date, A., Yoshida, T., Afzal, U., Kanvinde, P., Babu, T., et al. (2017). Preclinical evaluation of intravesical cisplatin nanoparticles for

non-muscle-invasive bladder cancer. *Clin. Cancer Res.* 23, 6592–6601. doi: 10.1158/1078-0432.CCR-17-1082

Kato, T., Bilim, V., Yuuki, K., Naito, S., Yamanobe, T., Nagaoka, A., et al. (2010). Bacillus Calmette-Guérin and BCG cell wall skeleton suppressed viability of bladder cancer cells *in vitro*. *Anticancer Res.* 30, 4089–4096.

Kresowik, T. (2009). Bacillus Calmette-Guerin immunotherapy for urothelial carcinoma of the bladder. *Immunotherapy* 1, 281–288. doi: 10.2217/1750743X.1.2.281

Lamm, D. L., and Allaway, M. (2000). Current trends in bladder cancer treatment. *Ann. Chir. Gynaecol.* 89, 234–241.

Li, L.-C., Okino, S. T., Zhao, H., Pookot, D., Place, R. F., Urakami, S., et al. (2006). Small dsRNAs induce transcriptional activation in human cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17337–17342. doi: 10.1073/pnas.0607015103

Lightfoot, A. J., Rosevear, H. M., and O'Donnell, M. A. (2011). Recognition and treatment of BCG failure in bladder cancer. *ScientificWorldJournal* 11, 602–613. doi: 10.1100/tsw.2011.30

Lu, S., Xu, L., Kang, E. T., Mahendran, R., Chiong, E., and Neoh, K. G. (2016). Co-delivery of peptide-modified cisplatin and doxorubicin via mucoadhesive nanocapsules for potential synergistic intravesical chemotherapy of non-muscle-invasive bladder cancer. *Eur. J. Pharm. Sci.* 84, 103–115. doi: 10.1016/j.ejps.2016.01.013

Malmström, P. U., Sylvester, R. J., Crawford, D. E., Friedrich, M., Krege, S., Rintala, E., et al. (2009). An individual patient data meta-analysis of the long-term outcome of randomised studies comparing intravesical mitomycin C versus Bacillus Calmette-Guérin for non-muscle-invasive bladder cancer. *Eur. Urol.* 56, 247–256. doi: 10.1016/j.eururo.2009.04.038

McKiernan, J. M., Barlow, L. J., Laudano, M. A., Mann, M. J., Petrylak, D. P., and Benson, M. C. (2011). A phase I trial of intravesical nanoparticle albumin-bound paclitaxel in the treatment of bacillus Calmette-Guérin refractory nonmuscle invasive bladder cancer. *J. Urol.* 186, 448–451. doi: 10.1016/j.juro.2011.03.129

Mckiernan, J. M., Holder, D. D., Ghandour, R. A., Barlow, L. J., Ahn, J. J., Kates, M., et al. (2014). Phase II trial of intravesical nanoparticle albumin bound paclitaxel for the treatment of nonmuscle invasive urothelial rin carcinoma of the bladder after bacillus Calmette-Guérin treatment failure. *J. Urol.* 192, 1633–1638. doi: 10.1016/j.juro.2014.06.084

Melamed, J. R., Riley, R. S., Valcourt, D. M., Billingsley, M. M., Kreuzberger, N. L., and Day, E. S. (2017). Quantification of siRNA duplexes bound to gold nanoparticle surfaces. *Methods Mol. Biol.* 1570, 1–15. doi: 10.1007/978-1-4939-6840-4_1

Miyazaki, J., Kawai, K., Kojima, T., Oikawa, T., Joraku, A., Shimazui, T., et al. (2011a). The liposome-incorporating cell wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guérin can directly enhance the susceptibility of cancer cells to lymphokine-activated killer cells through up-regulation of natural-killer group 2, member D ligands. *BJU Int.* 108, 1520–1526. doi: 10.1111/j.1464-410X.2010.10056.x

Miyazaki, J., Nishiyama, H., Yano, I., Nakaya, A., Kohama, H., Kawai, K., et al. (2011b). The therapeutic effects of R8-liposome-BCG-CWS on BBN-induced rat urinary bladder carcinoma. *Anticancer Res.* 31, 2065–2071.

Moch, H., Cubilla, A. L., Humphrey, P. A., Reuter, V. E., and Ulbright, T. M. (2016). The 2016 WHO classification of tumours of the urinary system and male genital organs—part A: renal, penile, and testicular tumours. *Eur. Urol.* 70, 93–105. doi: 10.1016/j.eururo.2016.02.029

Morales, A., Eidinger, D., and Bruce, A. W. (1976). Intracavitary bacillus Calmette-Guerin in the treatment of superficial bladder tumors. *J. Urol.* 116, 180–182. doi: 10.1016/S0022-5347(17)58737-6

Morales, A., Phadke, K., and Steinhoff, G. (2009). Intravesical mycobacterial cell wall-DNA complex in the treatment of carcinoma *in situ* of the bladder after standard intravesical therapy has failed. *J. Urol.* 181, 1040–1045. doi: 10.1016/j.juro.2008.11.019

Müller, K., Klein, P. M., Heissig, P., Roidl, A., and Wagner, E. (2016). EGF receptor targeted lipo-oligocation polyplexes for antitumoral siRNA and miRNA delivery. *Nanotechnology* 27, 1–15. doi: 10.1088/0957-4484/27/46/464001

Nakamura, T., Fukiage, M., Higuchi, M., Nakaya, A., Yano, I., Miyazaki, J., et al. (2014a). Nanoparticulation of BCG-CWS for application to bladder cancer therapy. *J. Control. Release* 176, 44–53. doi: 10.1016/j.jconrel.2013.12.027

Nakamura, T., Fukiage, M., Suzuki, Y., Yano, I., Miyazaki, J., Nishiyama, H., et al. (2014b). Mechanism responsible for the antitumor effect of BCG-CWS using the LEEL method in a mouse bladder cancer model. *J. Control. Release* 196, 161–167. doi: 10.1016/j.jconrel.2014.10.007

Nowicka, A. M., Kowalczyk, A., Jarzebinska, A., Donten, M., Krysinski, P., Stojek, Z., et al. (2013). Progress in targeting tumor cells by using drug-magnetic nanoparticles conjugate. *Biomacromolecules* 14, 828–833. doi: 10.1021/bm301868f

Pan, J., Li, X., Wu, W., Xue, M., Hou, H., Zhai, W., et al. (2016). Long non-coding RNA UCA1 promotes cisplatin/gemcitabine resistance through CREB modulating miR-196a-5p in bladder cancer cells. *Cancer Lett.* 382, 64–76. doi: 10.1016/j.canlet.2016.08.015

Poletajew, S., Zapała, P., and Radziszewski, P. (2017). Safety and efficacy of intravesical Bacillus Calmette-Guérin immunotherapy in patients with non-muscle-invasive bladder cancer presenting with asymptomatic bacteriuria: a systematic review. *Urol. Int.* 99, 1–5. doi: 10.1159/000477673

Rebouissou, S., Bernard-Pierrot, I., de Reyniès, A., Lepage, M.-L., Krucker, C., Chapeaublanc, E., et al. (2014). EGFR as a potential therapeutic target for a subset of muscle-invasive bladder cancers presenting a basal-like phenotype. *Sci. Transl. Med.* 6:244ra91. doi: 10.1126/scitranslmed.3008970

Rezaei, G., Habibi-Anbouhi, M., Mahmoudi, M., Azadmanesh, K., Moradi-Kalbolandi, S., Behdani, M., et al. (2017). Development of anti-CD47 single-chain variable fragment targeted magnetic nanoparticles for treatment of human bladder cancer. *Nanomedicine* 12, 597–613. doi: 10.2217/nnm-2016-0302

Rübben, H., Lutzeyer, W., Fischer, N., Deutz, F., Lagrange, W., and Giani, G. (1988). Natural history and treatment of low and high risk superficial bladder tumors. *J. Urol.* 139, 283–285. doi: 10.1016/S0022-5347(17)42387-1

Seth, S., Matsui, Y., Fosnaugh, K., Liu, Y., Vaish, N., Adami, R., et al. (2011). RNAi-based therapeutics targeting survivin and PLK1 for treatment of bladder cancer. *Mol. Ther.* 19, 928–935. doi: 10.1038/mt.2011.21

Shelley, M. D., Kynaston, H., Court, J., Wilt, T. J., Coles, B., Burgon, K., et al. (2001). A systematic review of intravesical bacillus Calmette-Guérin plus transurethral resection vs transurethral resection alone in Ta and T1 bladder cancer. *BJU Int.* 88, 209–216. doi: 10.1046/j.1464-410x.2001.02306.x

Skinner, E. C., Goldman, B., Sakr, W. A., Petrylak, D. P., Lenz, H. J., Lee, C. T., et al. (2013). SWOG S0353: Phase II trial of intravesical gemcitabine in patients with nonmuscle invasive bladder cancer and recurrence after 2 prior courses of intravesical bacillus calmette-guérin. *J. Urol.* 190, 1200–1204. doi: 10.1016/j.juro.2013.04.031

Sobin, L., and Gospodarowicz, M., W. C., (eds.). (2009). *TNM: Classification of Malignant Tumours*. Oxford, UK: Wiley-Blackwell.

Stapf, M., Teichgräber, U., and Hilger, I. (2017). Methotrexate-coupled nanoparticles and magnetic nanochemothermia for the relapse-free treatment of T24 bladder tumors. *Int. J. Nanomedicine* 12, 2793–2811. doi: 10.2147/IJN.S120969

Steinberg, G., Bahnson, R., Brosman, S., Middleton, R., Wajsman, Z., and Wehle, M. (2000). Efficacy and safety of valrubicin for the treatment of Bacillus Calmette-Guerin refractory carcinoma *in situ* of the bladder. The Valrubicin Study Group. *J. Urol.* 163, 761–767. doi: 10.1016/S0022-5347(05)67799-3

Sudha, T., Bharali, D. J., Yalcin, M., Darwish, N. H., Coskun, M. D., Keating, K. A., et al. (2017). Targeted delivery of cisplatin to tumor xenografts via the nanoparticle component of nano-diamino-tetrac. *Nanomedicine* 12:nnm-2016-0315. doi: 10.2217/nnm-2016-0315

Sweeney, A. E. (2015). Nanomedicine concepts in the general medical curriculum: initiating a discussion. *Int. J. Nanomed.* 10, 7319–7331. doi: 10.2147/IJN.S96480

Sylvester, R. J. (2008). Editorial comment on: prognostic factors in patients with non-muscle-invasive bladder cancer treated with bacillus Calmette-Guérin: multivariate analysis of data from four randomized CUETO trials. *Eur. Urol.* 53, 1002. doi: 10.1016/j.eururo.2007.10.007

Sylvester, R. J., van der Meijdena, A. P., and Lamm, D. L. (2002). Intravesical bacillus Calmette-Guerin reduces the risk of progression in patients with superficial bladder cancer: a meta-analysis of the published results of randomized clinical trials. *J. Urol.* 168, 1964–1970. doi: 10.1016/S0022-5347(05)64273-5

Uenishi, Y., Kawabe, K., Nomura, T., Nakai, M., and Sunagawa, M. (2009). Morphological study on *Mycobacterium bovis* BCG Tokyo 172 cell wall skeleton (SMP-105). *J. Microbiol. Methods* 77, 139–144. doi: 10.1016/j.mimet.2009.01.019

Uenishi, Y., Okada, T., Okabe, S., and Sunagawa, M. (2007). Study on the cell wall skeleton derived from *Mycobacterium bovis* BCG Tokyo 172 (SMP-105): establishment of preparation and analytical methods. *Chem. Pharm. Bull. (Tokyo)* 55, 843–852. doi: 10.1248/cpb.55.843

van der Heijden, A. G., Kiemeney, L. A., Gofrit, O. N., Nativ, O., Sidi, A., Leib, Z., et al. (2004). Preliminary European results of local microwave hyperthermia and chemotherapy treatment in intermediate or high risk superficial transitional cell carcinoma of the bladder. *Eur. Urol.* 46, 65–71; discussion 71–72. doi: 10.1016/j.eururo.2004.01.019

Wang, X., Zhang, F., and Wu, X.-R. (2017). Inhibition of pyruvate kinase M2 markedly reduces chemoresistance of advanced bladder cancer to cisplatin. *Sci. Rep.* 7:45983. doi: 10.1038/srep45983

Wei, Y., Gao, L., Wang, L., Shi, L., Wei, E., Zhou, B., et al. (2017). Polydopamine and peptide decorated doxorubicin-loaded mesoporous silica nanoparticles as a targeted drug delivery system for bladder cancer therapy. *Drug Deliv.* 24, 681–691. doi: 10.1080/10717544.2017.1309475

Xin, Y., Huang, M., Guo, W. W., Huang, Q., Zhang, L. Z., and Jiang, G. (2017). Nano-based delivery of RNAi in cancer therapy. *Mol. Cancer* 16, 134. doi: 10.1186/s12943-017-0683-y

Yang, X., Su, L.-J., La Rosa, F. G., Smith, E. E., Schlaepfer, S. K., Cho, S. K., et al. (2015). The antineoplastic activity of photothermal ablative therapy with targeted gold nanorods in an orthotopic urinary bladder. *Bladder Cancer* 3, 201–210. doi: 10.3233/BLC-170096

Yao, S., Li, X., Liu, J., Sun, Y., Wang, Z., and Jiang, Y. (2017). Maximized nanodrug-loaded mesenchymal stem cells by a dual drug-loaded mode for the systemic treatment of metastatic lung cancer. *Drug Deliv.* 24, 1372–1383. doi: 10.1080/10717544.2017.1375580

Yurgel, V. C., Oliveira, C. P., Begnini, K. R., Schultze, E., Thurow, H. S., Leon, P. M. M., et al. (2014). Methotrexate diethyl ester-loaded lipid-core nanocapsules in aqueous solution increased antineoplastic effects in resistant breast cancer cell line. *Int. J. Nanomed.* 9, 1583–1591. doi: 10.2147/IJN.S56506

Zhang, D., Sun, P., Li, P., Xue, A., Zhang, X., Zhang, H., et al. (2013). A magnetic chitosan hydrogel for sustained and prolonged delivery of Bacillus Calmette-Guérin in the treatment of bladder cancer. *Biomaterials* 34, 10258–10266. doi: 10.1016/j.biomaterials.2013.09.027

Zhang, Q., Neoh, K. G., Xu, L., Lu, S., Kang, E. T., Mahendran, R., et al. (2014). Functionalized mesoporous silica nanoparticles with mucoadhesive and sustained drug release properties for potential bladder cancer therapy. *Langmuir* 30, 6151–6161. doi: 10.1021/la500746e

Zhu, R., Wang, Z., Liang, P., He, X., Zhuang, X., Huang, R., et al. (2017). Efficient VEGF targeting delivery of DOX using Bevacizumab conjugated SiO 2 @LDH for anti-neuroblastoma therapy. *Acta Biomater.* 63, 163–180. doi: 10.1016/j.actbio.2017.09.009

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REVIEW
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Modulating the Tumor Microenvironment to Enhance Tumor Nanomedicine Delivery

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Nanomedicines including liposomes, micelles, and nanoparticles based on the enhanced permeability and retention (EPR) effect have become the mainstream for tumor treatment owing to their superiority over conventional anticancer agents. Advanced design of nanomedicine including active targeting nanomedicine, tumor-responsive nanomedicine, and optimization of physicochemical properties to enable highly effective delivery of nanomedicine to tumors has further improved their therapeutic benefits. However, these strategies still could not conquer the delivery barriers of a tumor microenvironment such as heterogeneous blood flow, dense extracellular matrix, abundant stroma cells, and high interstitial fluid pressure, which severely impaired vascular transport of nanomedicines, hindered their effective extravasation, and impeded their interstitial transport to realize uniform distribution inside tumors. Therefore, modulation of tumor microenvironment has now emerged as an important strategy to improve nanomedicine delivery to tumors. Here, we review the existing strategies and approaches for tumor microenvironment modulation to improve tumor perfusion for helping more nanomedicines to reach the tumor site, to facilitate nanomedicine extravasation for enhancing transvascular transport, and to improve interstitial transport for optimizing the distribution of nanomedicines. These strategies may provide an avenue for the development of new combination chemotherapeutic regimens and reassessment of previously suboptimal agents.

Keywords: tumor microenvironment, nanomedicine, tumor nanomedicine delivery, interstitial fluid pressure, tumor perfusion, extracellular matrix

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INTRODUCTION

In recent times, nanomedicine delivery to tumors has attracted extensive attention in the field of tumor treatment (Allen and Cullis, 2004; Peer et al., 2007). The advantage of nanomedicines over free drugs is based on the enhanced permeability and retention (EPR) effect (Fang et al., 2011; Maeda, 2012). The fundamental characteristics of EPR physiology are highly permeable tumor vessels allowing the enhanced permeability (EP) of large particles including proteins, macromolecules, liposomes, micelles, and other particles large enough to avoid renal clearance, into the tumor interstitium combined with impaired lymphatic drainage limiting clearance and causing enhanced retention (ER) of those extravasated particles. Both features result from the rapid growth of a tumor and collapse of the existing blood

and lymph vessels in the limited interstitial space (Leu et al., 2000; Dreher et al., 2006). With EPR effect as the main principle for passive targeting strategy, nanomedicine delivery to tumors has achieved success to varying degrees. However, the clinical benefits of the three EPR-based Food and Drug Administration (FAD)-approved nanomedicines including pegylated liposomal doxorubicin (Doxil/Caelyx), liposomal daunorubicin (DaunoXome), and nanoparticle albumin-bound paclitaxel (Abraxane) for the treatment of solid tumors were demonstrated to be only modest (O'Brien et al., 2004; Gradishar et al., 2005; Jain and Stylianopoulos, 2010), posing considerable challenges for the clinical translation of new nanomedicines. Accumulating evidence revealed that EPR-dependent drug delivery was always compromised by the tumor microenvironment including irregular vascular distribution, elevated tumor interstitial fluid pressure (IFP), poor blood flow, rich extracellular matrix (ECM) and abundant tumor stroma cells (Nichols and Bae, 2014). Delivery barriers posed by the tumor microenvironment are the main reasons responsible for the modest survival benefits of FDA-approved nanomedicines (Jain and Stylianopoulos, 2010).

The tumor microenvironment, as an important component of tumor tissues, functions as the soil for the seeds i.e., tumor cells to proliferate, differentiate, and promote tumor growth (Zhang et al., 2015, 2016a). The components of tumor microenvironment include the extracellular matrix (ECM) and different kinds of stromal cells such as tumor-associated fibroblasts (TAF), tumor-associated macrophages, and pericytes (Hanahan and Weinberg, 2011). As a pathologic condition, the tumor microenvironment is remarkably abnormal: tumor blood flow is low, perfusion is uneven in tumors, the tumor vessel permeability is highly heterogeneous, interstitial fluid pressure (IFP) is elevated, and a large number of active stromal cells and ECM are often dense and stiff.

Systemically administrated nanomedicines need to undergo a three-step process in solid tumors to achieve their therapeutic effect: vascular transportation to different areas of the tumor, trans-vascular transport across the vessel wall, and interstitial transport to reach the tumor cells (Wong et al., 2011). Delivery of nanomedicines differs markedly between tumors and normal tissues owing to structural differences. The abnormality in organization and structure of the tumor vasculature leads to heterogeneous blood flow, which directly influences the vascular transport of nanomedicines (Jain, 1994, 2005). Additionally, the vascular hyper-permeability and lack of functional lymphatic vessels inside tumors results in elevated IFP (Boucher et al., 1990), which not only compresses tumor vessels to aggravate the heterogeneous blood flow, but also reduces convective transport of nanomedicines (Jain, 1987). Besides, compression from proliferating tumor cells, stromal cells, and the ECM could also compress tumor vessels (Stylianopoulos et al., 2012). Furthermore, the dense ECM hinders interstitial diffusion of nanomedicines (Jain, 1987). When 90-nm liposomes, approximately the size of liposomal doxorubicin, an approved nanomedicine, was intravenously administered in tumor-bearing mice, these particles leaked out of tumor vessels but did not move far away from the vessel wall (Yuan et al., 1994a). Even directly intra-tumor injected 150-nm particles did not move far from the injection site (McKee et al., 2006). Altogether, the complex tumor microenvironment could negatively affect vascular transport, trans-vascular transport and interstitial transport of nanomedicine, and compromise nanomedicine delivery for tumor treatment.

To improve the therapeutic benefits of nanomedicine, different strategies including active targeting nanomedicine (Zhang et al., 2014a,b), tumor-responsive nanomedicine (Zhu et al., 2012; Huang et al., 2013), as well as optimization of the physiochemical parameters of nanomedicine such as size (Tong et al., 2013; Tang et al., 2014), charge (Han et al., 2015), and shape (Chauhan et al., 2011) have been developed. However, these methods rely on the advanced development of the nanomedicine itself, which could not conquer the above-mentioned delivery barriers of the tumor microenvironment (Chauhan and Jain, 2013). Accordingly, a modification of the tumor microenvironment was recognized as an important tool to improve tumor nanomedicine delivery (Jain and Stylianopoulos, 2010; Miao et al., 2015). In this review, in terms of the three processes including vascular transport, trans-vascular transport and interstitial transport that nanomedicines need to experience before reaching tumor cells and achieving therapeutic benefit, we tried to summarize different strategies of modulating tumor microenvironment to improve tumor nanomedicine delivery from the corresponding three aspects including improving tumor perfusion, facilitating nanomedicine extravasation, and enhancing interstitial transport of nanomedicine.

NANOMEDICINE TRANSPORT BARRIERS FROM TUMOR MICROENVIRONMENT

The main transport barriers of a tumor microenvironment include abnormal tumor vasculature, elevated IFP, dense ECM, and stromal cells (**Figure 1**). These components varied with respect to different tumor types. To better understand and address the complexities of a tumor microenvironment, we used two representative models—highly permeable tumors and highly desmoplastic tumors (Stylianopoulos and Jain, 2013). Highly permeable tumors such as gliomas and melanomas are always rich in vessels and a small amount of pericytes, TAFs, and ECM, wherein tumor cells are in the vicinity of tumor vessels. On the other hand, highly desmoplastic tumors such as pancreatic cancers (Cabral et al., 2011), bladder cancers (Zhang et al., 2014), and some breast cancers (Stylianopoulos and Jain, 2013) are always hypovascular with numerous TAFs, a dense ECM, and high coverage rate of pericytes on the endothelium, such that the tumor cells are isolated into nests by TAF complexed with ECM and are a certain distance from the tumor vessels (Feig et al., 2012; Zhang et al., 2016c). Highly permeable and highly desmoplastic tumors were also referred to as tumors with tumor-vessels architecture and tumors with stroma-vessels architecture, respectively, in some reports (Smith et al., 2013; Miao et al., 2016).

Abnormal Tumor Vasculature Networks

In normal tissues, an exquisite counterbalance is achieved between the proangiogenic molecules such as VEGF and endogenous antiangiogenic molecules such as sVEGFR1 and

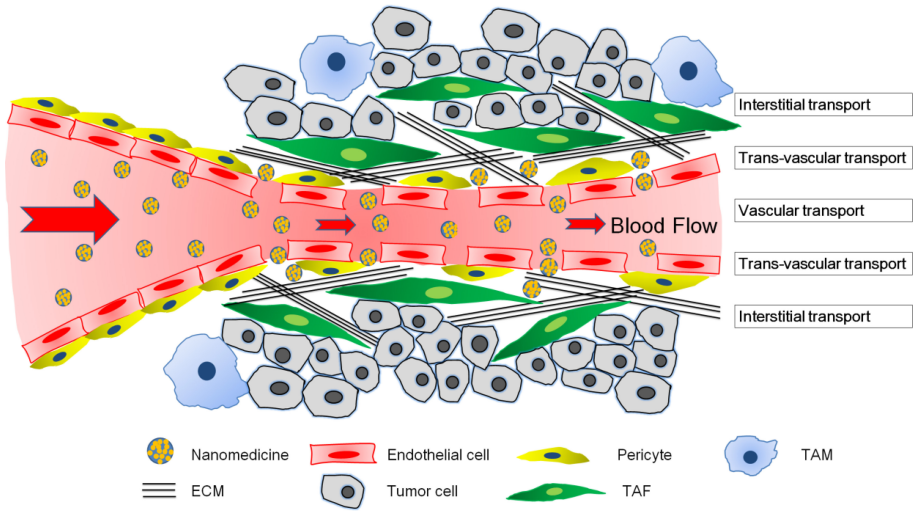

FIGURE 1 | The transport barriers for tumor nanomedicine delivery imposed by a complicated tumor microenvironment including poor blood perfusion, IFP, dense ECM, and a large number of stromal cells. The nanomedicines have to cross the blood vessel walls, penetrate the extravascular space, and eventually reach tumor cells to exert their therapeutic effects.

thrombospondins (Carmeliet and Jain, 2000; Jain, 2003). In tumor tissues, however, the proangiogenic effect is abnormally upregulated and the pathological angiogenesis occurs in a disorganized manner. Compared to normal vessels, tumor vessels are highly irregular and chaotic in structure with wide endothelial gaps and a heterogeneous basement membrane (Carmeliet and Jain, 2011). The tortuous and leaky nature of tumor vessels contributes to compromised tumor blood flow, as the high tortuosity of tumor vessels results in an elevated geometric resistance that retards the blood flow (Sevick and Jain, 1989a). Furthermore, this sharp drop of blood flow due to the geometric resistance exerts a marked influence on the viscous resistance of blood in tumor vessels (Sevick and Jain, 1989b). Additionally, the leaky nature of tumor vessels allows the permeability of fluid into the interstitium. Fluid loss would increase the hematocrit of tumor blood to elevate its viscosity and further impede tumor blood flow (Sevick and Jain, 1989b; Netti et al., 1996; Sun et al., 2007). This combined effect of tortuosity of and leakage from tumor vessels compromises blood flow in brain tumors to one to three orders of magnitude, slower than that in the pial vessels surrounding normal tissues (Yuan et al., 1994b). Inefficient blood flow in tumors always leads to poor delivery of systemically administered drugs. Therefore, the tumor vascular network poses a major barrier in vascular transportation of nanomedicine, specifically highly permeable tumors with a variety of hyper permeable vessels.

Elevated IFP

IFP is a type of stress exerted by fluids and is uniformly elevated throughout the tumor bulk in many tumors. Fluid flow is involved in three processes including flow along the tumor vessels, through the tumor interstitium, and drainage of excessive fluid by the lymphatic vessels (Jain et al., 2014).

Abnormalities in the tumor microenvironment concerning these three processes lead to elevated IFP. The leaky tumor vessels allow the extravasation of excessive fluid and plasma macromolecules into the tumor interstitium. In normal tissues, excessive fluid could be drained by an effective lymphatic network to maintain a balanced tissue interstitial pressure. However, lymphatic drainage in tumors do not function properly and hence, IFP is elevated in tumor tissues. Apart from abnormality in tumor blood vessels and lymphatic vessels, abnormal hydraulic conductivity is also a regulator of IFP, especially in highly desmoplastic tumors. The hydraulic conductivity depends on the volume fraction, surface charge, chemical composition, and organization of fibers in the tumor interstitial space (Levick, 1987; Stylianopoulos et al., 2008). Tumors abundant in collagen could display an order of magnitude lower in hydraulic conductivity than those with low collagen content (Netti et al., 2000). The negatively charged glycosaminoglycans could increase flow resistance because of their ability to trap water (Levick, 1987). Therefore, depletion of glycosaminoglycans with matrix metalloproteinases-1 and -8 increases the hydraulic conductivity and thus, the interstitial fluid velocity (Mok et al., 2007). Furthermore, a high density of tumor cells and stromal cells might reduce the interstitial space available for fluid flow, thereby increasing fluid resistance and IFP. In normal tissues, IFP is in the range of 0–3 mm Hg. However, experimental and human solid tumors exhibit high IFP, typically ranging from 5 to 40 mmHg, which may reach 75–130 mmHg in highly desmoplastic pancreatic tumors (Milosevic et al., 2004; Provenzano et al., 2012). Therefore, in many tumors, elevated IFP and reduced microvascular pressure (MVP) hinder nanomedicine delivery by the following mechanisms. First, as IFP and MVP also impose fluid stresses on vessel walls, elevated IFP could compress tumor vessels to cause blood stasis and/or vessel collapse to reduce the vascular

transport of nanomedicine. Another consequence of high IFP is that interstitial fluid might escape from the tumor periphery into surrounding normal tissue, carrying not only nanomedicine to reduce the trans-vascular transport of nanomedicine but also growth factors or tumor cells to drive tumor metastasis or drug resistance (Chary and Jain, 1989; Netti et al., 1996). Finally, IFP might force the nanomedicine to extravasate by passive diffusion, instead of convention, a much faster transport process, which compromises the interstitial transport of nanomedicine especially larger nanomedicines. To conclude, elevated IFP inside tumors hinders vascular, trans-vascular, and interstitial transport of nanomedicines.

ECM

The ECM is the non-cellular component widely present within all tissues and organs. ECM is mainly composed of two types of macromolecules: proteoglycans (PGs) and fibrous proteins (Järveläinen et al., 2009; Schaefer and Schaefer, 2010). PGs such as hyaluronan fill the majority of the extracellular interstitium of the tissue in the form of a hydrated gel. The fibrous ECM proteins include collagens, elastins, fibronectins, and laminins (Dequidt et al., 2007). ECM is a highly dynamic structure being constantly remodeled, either enzymatically or non-enzymatically, and its final components are controlled by a myriad of post-translational modifications. ECM is tissue-specific and the component varies greatly among different tissues including cancerous ones (Frantz et al., 2010).

Under normal conditions, the unique composition and structure of the ECM functions as a growth regulator. ECM and ECM-associated enzymes and growth factors regulate cell proliferation and differentiation, maintaining cell survival and dynamic homeostasis (Li et al., 2010). However, ECM is commonly deregulated and becomes disorganized in diseases such as cancer. Fibrosis due to excessive ECM production or limited ECM turnover occurs in many types of cancers. Especially in highly desmoplastic tumor such as pancreatic cancer and some breast cancers (Stylianopoulos and Jain, 2013), a dense ECM composed of collagen, hyaluronan, and fibronectin is always found (Feig et al., 2012; Zhang et al., 2016c). In contrast, tumors with abundant vessels always harbor a low level of ECM. Signaling pathways involved in ECM production included transforming growth factor-beta (TGF-β), Hedgehog (Hh) signaling, and platelet-derived growth factor (PDGF). ECM turnover is subjected to enzyme-mediated remolding including heparanase, cysteine proteases, 6-O-sulfatases, urokinase, and many matrix metalloproteinases (MMPs) (Egeblad et al., 2010; Lu et al., 2012). In highly vascularized and permeable tumors such as glioma and melanoma, ECM is always scarce.

The dense ECM in the tumor interstitium not only compressed tumor vasculature and reduced vascular transport of nanomedicine but also isolates tumor cells into nests within a certain distance from collapsed vessels and resists the free penetration and homogeneous distribution of nanomedicine in three main ways (Bailey et al., 2008; Miao et al., 2015). First, limited interstitial volume plus high stromal fraction and large matrix molecules result in a dense network (Padera et al., 2004), effectively reducing blood flow and limiting convection of nanomedicine. Second, the fibrillar structure, mesh size, and collagen thickness directly limit the diffusion of nanomedicine. The diffusion capacity is inversely related to the size of nanomedicine. Matrix mesh size ranges between 20 and 40 nm in solid tumors. Particles larger than the mesh size are completely prevented from diffusing through the ECM, those near the mesh size can be hindered to a certain extent, and only small particles can penetrate almost freely (Nichols and Bae, 2012). Third, the tortuous nature of the interstitial space poses an additional barrier for drugs of all size, because it elongates the diffusion path of the nanomedicine from blood vessels to tumor cells (Chauhan et al., 2009). The resistance of nanomedicine delivery from ECM mainly occurs in highly desmoplastic tumors. For highly permeable tumors with tumor-vessels architecture, the ECM is not as abundant, dense, and stiff as that in highly desmoplastic tumors, the and nanomedicine can penetrate throughout the tumor tissues much more easily after its extravasation from tumor vessels (Cabral et al., 2011). Unfortunately, the ECM is much more denser and thicker in human tumors than in mouse models (Miao and Huang, 2015). In conclusion, rich ECM in tumors resists vascular and interstitial transport of nanomedicine.

Stromal Cells

Stromal cells include TAF, tumor-associated macrophages (TAM), and pericytes. The origin of TAF is still debatable. TAF probably originated from resident tissue fibroblasts, bone marrow-derived mesenchymal stem cells, hematopoietic stem cells, epithelial cells (epithelial-mesenchymal transition; EMT), and endothelial cells (endothelial-mesenchymal transition; EndMT) (Shiga et al., 2015). It was now widely accepted that TAF significantly contributes to cancer progression (Brennen et al., 2012). TAF is abundant in highly desmoplastic tumors and produces large amounts of ECM to isolate tumor cells into a nest. TAF has been regarded as the major component of tumor stroma and contributes to the binding-site barrier for interstitial transport of nanomedicine (Miao et al., 2016). Large numbers of TAF associated with dense ECM also compress tumor vessels to compromise the vascular transport of nanomedicine. Besides, reports have shown that uptake of the anisamide ligand-modified nanomedicine by TAF was 7-fold higher than that of the other cells because of the different expression level of the sigma receptor between TAF and other cells (Miao et al., 2016).

TAM is the major cancer-related inflammatory cell primarily converted from monocytes that are closely associated with the prognosis of many cancer types. Other inflammatory cells include granulocytes, dendritic cells, and myeloid derived suppressor cells, which are also important constituents of the tumor microenvironment (Mocellin et al., 2001; Hu et al., 2016). When polarized toward the anti-inflammatory state by the tumor microenvironment, TAM promotes immune evasion and angiogenesis, thereby driving tumor growth (Cieslewicz et al., 2013). The off-target effect of nanomedicines is inevitable because of the phagocytic properties of inflammatory cells. Roode et al. showed that the association between TAM and NP were 4-fold greater than that of cancer cells despite TAM constituting only 1% of all cells in tumors (Roode et al., 2016). The off-target uptake

of nanomedicine by stromal cells including TAF and TAM could certainly reduce the uptake of nanomedicine by tumor cells and therefore the therapeutic benefits.

Pericytes are another important type of stromal cells located mainly in the perivascular space, which also affect nanomedicine delivery. Neither leaky, immature vessels with little coverage nor over-mature vessels with high pericyte coverage are favorable for nanomedicine delivery. Excessively leaky vessels in highly vascularized tumors affect nanomedicine delivery mainly by compromising blood perfusion and thereby the vascular transport of nanomedicine (Stylianopoulos and Jain, 2013). In contrast, high pericyte coverage is always found in highly desmoplastic tumors, which reduce the endothelial gap and limit the trans-vascular transport of nanomedicine, especially for larger nanomedicine (Cabral et al., 2011).

STRATEGIES TO MODULATE TUMOR MICROENVIRONMENT

In accordance with the three processes including vascular, trans-vascular and interstitial transport that nanomedicines need to experience before reaching tumor cells, strategies of modulating tumor microenvironment to improve nanomedicine delivery for tumor treatment can be divided into three categories: improving tumor perfusion, facilitating nanomedicine extravasation, and enhancing interstitial transport of nanomedicine (**Table 1**).

Improving Tumor Perfusion

Tumor Vasculature Normalization

The newly formed tumor vessels are always tortuous and leaky allowing the extravasation of nanomedicine but simultaneously increasing IFP, which prevents adequate and homogeneous blood flow and vascular transport of nanomedicine. To improve nanomedicine delivery for tumor treatment, normalization of vessels has emerged as an effective approach. Vessel normalization transforms the abnormal phenotype of tumor vessels into a phenotype that closely resembles that of fully functional normal vessels by repairing the basement membrane and increasing coverage rate of pericytes, and ultimately decreasing vessel leakiness. Optimizing the structure of tumor vessels could reduce the extravasation of excessive fluid and lower IFP, and then restore tumor blood flow, thereby improving vascular transport of nanomedicine. Many proangiogenic molecules including VEGF, fibroblast growth factor (FGF), and PDGF are overexpressed in tumors and involved in angiogenesis, which cause chaotic structural development in these newly formed tumor vessels (Goel et al., 2011). Therefore, strategies to block these proangiogenic signaling molecules were designed to repair tumor vessels. For example, VEGF inhibitors Bevacizumab, the FDA-approved antiangiogenic monoclonal antibody (mAb), capable of reverting abnormal structure of tumor vessels toward a more normal phenotype have been applied in the treatment of metastatic colorectal cancer (Salgaller, 2003; Ellis, 2005; **Table 1**), which were of high potential to improve nanomedicine delivery for tumor treatment. Moreover, some angiogenic signaling pathways such as mTOR signaling (Guo et al., 2014), Notch 1 signaling (Maes et al., 2014), and D2 receptors-angiopoietin 1 signaling (Chakroborty et al., 2011) involved in vessel normalization have also been modulated to improve nanomedicine delivery (**Table 1**). In our previous research, it was also shown imatinib mesylate (IMA) could normalize the tumor vessels of A549 tumors by inhibiting platelet-derived growth factor signaling pathway (Zhang et al., 2016d). Interestedly, IMA treatment could significantly reduce the accumulation of nanoparticles (NPs) around 110 nm but enhanced the accumulation of micelles around 23 nm. Furthermore, IMA treatment limited the distribution of NPs inside tumors but increased that of micelles with a more homogeneous pattern (**Figure 2**). Finally, the anti-tumor efficacy study displayed that IMA pretreatment could significantly increase the therapeutic effects of paclitaxel-loaded micelles. As tumor vessel normalization minimized endothelial gap, it could prevent tumor cells shedding into tumor vessels, and reduce the possible tumor metastasis to a certain degree.

To utilize vessel normalization strategy to improve nanomedicine delivery for tumor treatment, four concerns should be kept in mind. First, the strategy can only improve the delivery of small molecular weight drugs or relatively smaller nanomedicines ranging from 20 to 40 nm, but reduces the delivery of large nanomedicines around 100 nm as it decreases the endothelial gap of tumor vessels (Chauhan et al., 2012; Zhang et al., 2016d, 2017b). Second, the normalization is transient and the followed nanomedicine should be applied in the normalization window. Third, a judicious dose of vascular normalizer is highly recommended to prevent excessive pruning of tumor vessels, which might impair vascular efficiency and thus the delivery of concurrent therapy (Huang et al., 2012; Jain, 2013). Fourth, as vasculatures are always severely compressed in highly desmoplastic tumors and are refractory to vasculature normalizers (Smith et al., 2013), this strategy might only be used in tumors that are highly permeable but not highly desmoplastic, or at least combined with other strategies capable of reopening compressed vessels.

Tumor Vessel Dilation

Vasoconstrictive endothelin-1 (ET1) and its receptor ETA via which ET-1 mediates vasoconstriction are both abundant in tumor tissues for maintaining the contractile tone of tumor vessels. The expression level of ET1 and ETA in tumor vessels was 13-fold and 5-folder higher than that of size-matched normal vessels, respectively (Sonveaux et al., 2004). BQ123, a selective antagonist against ETA could inhibit ET1-ETA signaling, induce tumor vessel dilation, and trigger a tumor-specific increase in blood flow. The blood flow improvement induced by BQ123 improved the delivery of free drugs to tumors despite an increase in IFP (Martinive et al., 2006). In addition, it was also demonstrated that BQ123 could increase the delivery of photothermal nanomedicine around 100 nm for effective photothermal therapy of tumors (Wang et al., 2017). Some inflammation factors such as bradykinin capable

TABLE 1 | Summary of tumor microenvironment modulation strategies for improving tumor nanomedicine delivery.

Modulation strategies	Main working mechanism	Modulation agents	Tumors	References
Improving tumor perfusion	Tumor vessel normalization by blocking tumor proangiogenic signaling	DC101 (VEGF mAb)	Mammary carcinoma, small cell lung carcinoma, glioblastoma multiforme, colon adenocarcinoma	Tong et al., 2004
		Bevacizumab (VEGF mAb)	Colon carcinoma, melanoma	Ellis, 2005; Turley et al., 2012
		SST0001 (Heparanase inhibitor)	Myeloma	Ritchie et al., 2011
		Rapamycin (mTOR signaling inhibition)	Melanoma	Guo et al., 2014
		Chloroquine (Notch 1 signaling inhibition)	Melanoma	Maes et al., 2014
		Dopamine (D2 receptors-angiopoietin 1 activation)	Prostate and colon tumor	Chakroborty et al., 2011
		Imatinib mesylate (PDGF signaling inhibition)	Lung carcinoma	Zhang et al., 2016d
	Tumor vessel dilation	BQ123 (ETA antagonist)	Colorectal carcinoma	Wang et al., 2017
		Captopril (hypotensor)	Glioma	Zhang et al., 2017a
Facilitating nanomedicine extravasation	Inflammatory mediators for enhancing vessel permeability	TNF- alpha	lymphoma and melanoma	Curnis et al., 2002; Seki et al., 2011
		Prostaglandin I2	Hepatocellular carcinoma	Tanaka et al., 2003
		VEGF	Glioma and colon carcinoma	Monsky et al., 1999
		Nitroglycerin (NG)	Sarcoma	Seki et al., 2009
	Pericyte depletion by inhibiting TGF signal pathway	A small-molecule TGF-β inhibitor, LY364947	Pancreatic cancer	Meng et al., 2013
		TGF- type I receptor (TR-I) inhibitor	Pancreatic cancer, gastric cancer	Kano et al., 2007
		ID11 (anti-TGF-β mAb)	Breast carcinoma	Liu et al., 2012
	Platelet depletion	Antiplatelet antibody R300	Breast cancer	Li et al., 2017
Enhancing interstitial transport	Direct ECM degradation	Matrix metalloproteinases-1 and−8	Sarcoma	Mok et al., 2007
		Hyaluronidase and hyaluronidase-loaded nanoparticles	Pancreatic cancer, breast cancer	Provenzano et al., 2012; Gong et al., 2016; Zhou et al., 2016
		PEGPH20 (PEGylated hyaluronidase)	Pancreatic cancer	Jacobetz et al., 2012; Hingorani et al., 2016
		rtPA	Lung cancer, melanoma	Zhang et al., 2016b; Kirtane et al., 2017
	ECM reduction by inhibiting TAF activity	IPI-926 (Hh inhibitor)	Pancreatic cancer	Olive et al., 2009
		Cyclopamine (Hh inhibitor)	Pancreatic cancer	Zhang et al., 2016c; Jiang et al., 2017
	TAF depletion or reprogramming	Losartan	Human breast, pancreatic, and skin tumors	Diop-Frimpong et al., 2011; Chauhan et al., 2013
		VDR ligand	Pancreatic cancer	Sherman et al., 2014
		ATAR	Pancreatic cancer	Froeling et al., 2011; Chronopoulos et al., 2016
		Quercetin nanoparticles downregulating Wnt16 expression	Bladder carcinoma	Hu et al., 2017

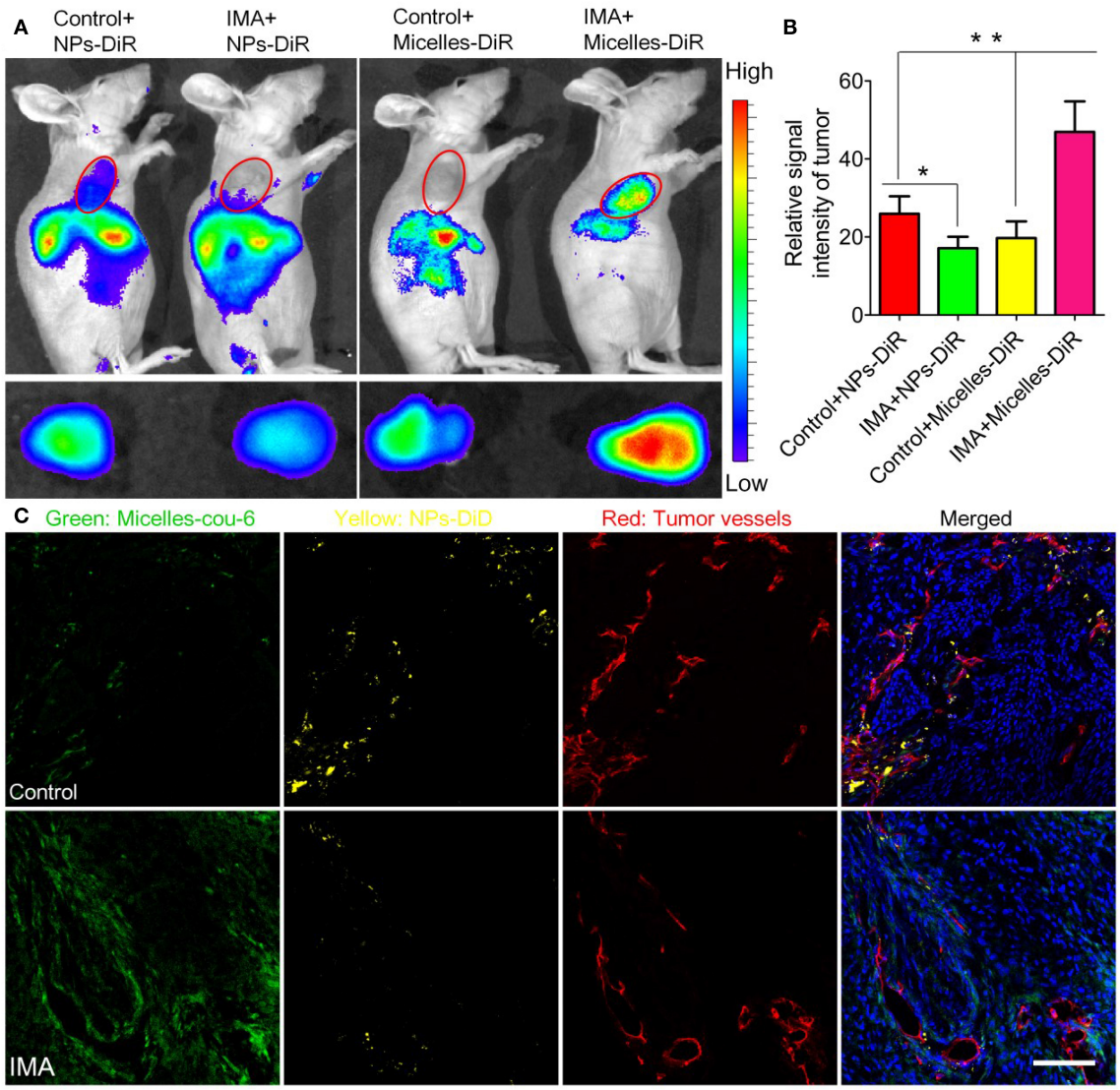

FIGURE 2 | The effects of IMA treatment on tumor nanoparticle delivery. **(A)** *In vivo* fluorescence imaging of A549 xenograft-bearing mice (the upper row) treated with IMA or water as a control, *ex vivo* fluorescence imaging of their corresponding tumor xenografts (the lower row), and **(B)** the relative signal intensity of tumor tissue 24 h post the injection of DiR-labeled nanoparticles or micelles. $^{*}p < 0.05$, compared with Control+NP group. $^{**}p < 0.01$ compared with IMA+Micelles group. **(C)** *In vivo* distribution of micelles and nanoparticles in tumor slices from A549 tumor xenograft-bearing mouse models treated with IMA or water at 24 h after i.v. injection of a mixture of DiD-labeled nanoparticles and coumarin-6-labeled micelles. The oral dose of IMA was 50 mg/kg/d for 3 weeks. The dose of both coumarin-6 and DiD was 0.05 mg/kg. The bar indicated 100 μm. Reprinted from reference with permission by copyright holder, Zhiqing Pang.

of dilating vessels could also directly increase tumor perfusion. In our previous study, it was shown captopril, a widely used hypotensor in clinics, could dilate tumor blood vessels by increasing bradykinin expression and even increase tumor vessel permeability to enhance nanomedicine delivery for tumor therapy (Zhang et al., 2017a).

Facilitating Nanomedicine Extravasation

Inflammatory Mediators for Enhanced Permeability

Inflammatory mediators such as TNFα (Seki et al., 2011), prostaglandin analogs (Tanaka et al., 2003), VEGF (Monsky et al., 1999), and nitric oxide (NO) donors (Seki et al., 2009), capable of enhancing vascular permeability, have been utilized to increase nanomedicine accumulation in tumors up to 2–6-fold higher than that of the control group (**Table 1**). Apart from vascular permeability enhancement, vasodilatation and blood-flow improvement by usage of inflammatory mediators were also involved in improving nanomedicine delivery for tumors. However, a series of effects of inflammatory mediators mentioned above could also lead to elevated IFP against nanomedicine delivery. Thus, accumulation of nanomedicine in tumors comprehensively depends on these factors. As inflammation might potentially promote cancer development (Atsumi et al., 2014), local application (Seki et al., 2011) or

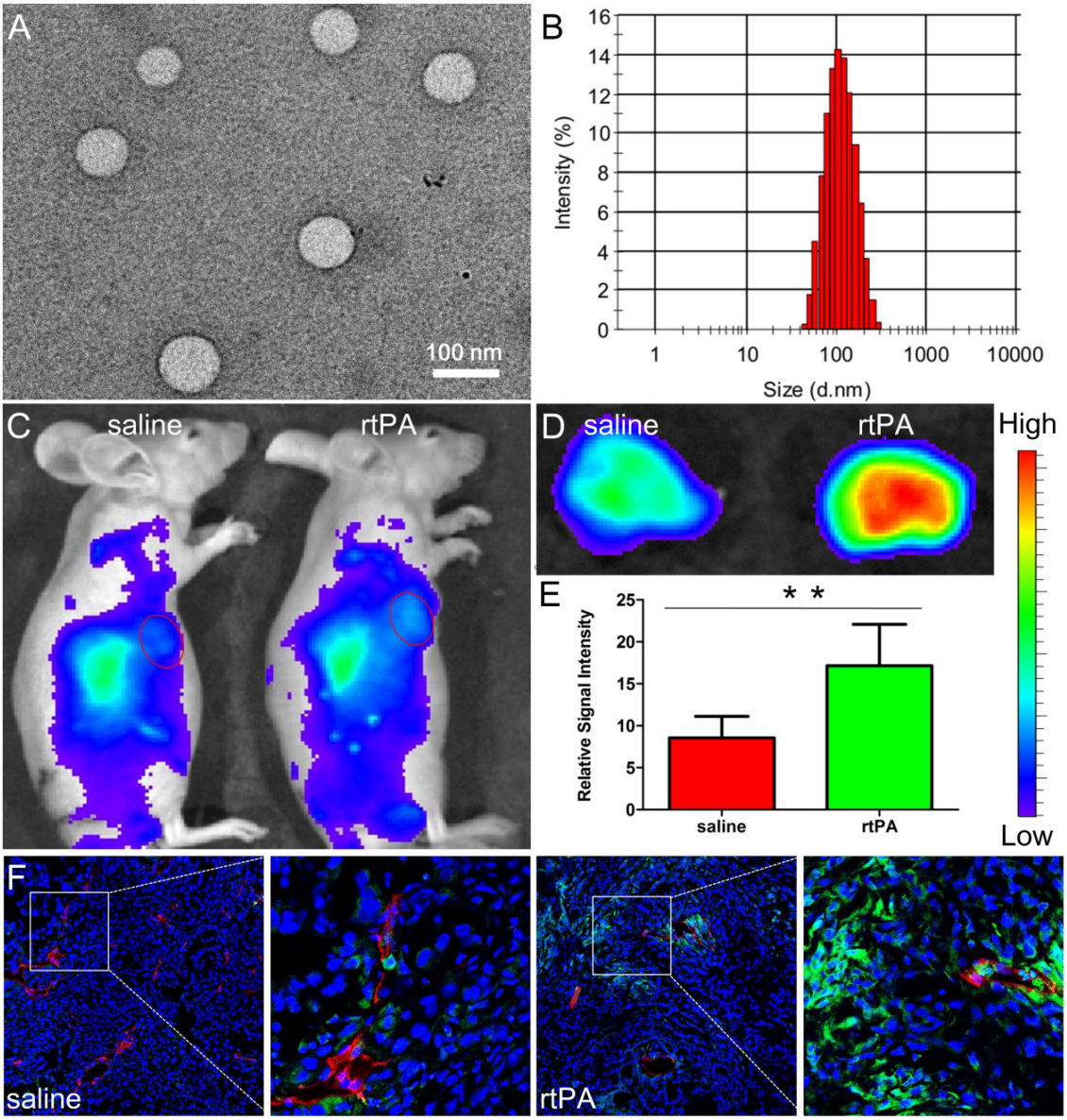

FIGURE 3 | Characterizations of NPs and effects of rtPA treatment on tumor nanoparticle delivery. TEM photograph **(A)** and size distribution **(B)** of NPs. Bar: 100 nm. *In vivo* **(C)** and *ex vivo* imaging **(D,E)** of A549 xenograft-bearing mice treated with 2 weeks of rtPA (25 mg/kg/d) or saline 24 h after the injection of DiR-labeled NPs. $^{**}p < 0.01$ rtPA vs. saline group. *In vivo* distribution of NPs in tumor tissues. **(F)** Original magnification: 120 ×. Reprinted from reference with permission, Copyright Elsevier, 2016.

targeted delivery of inflammatory mediators to the tumor site should be adopted.

Pericyte Depletion

In highly desmoplastic tumor, the coverage rate of pericytes on endothelium was about 70%, much higher than highly permeable tumors, which significantly limit the transvascular movement of nanomedicine into tumor interstium. Therefore, strategies by using low dose of a TGF-β inhibitor, LY364947 was developed to reduce the pericyte coverage of endothelium and increase size gaps between endothelium to increase therapeutic benefits of gemcitabine-loaded liposomes for pancreatic cancer (Cabral et al., 2011; Meng et al., 2013) and Doxil for diffuse-type gastric cancer (Kano et al., 2007).

Platelet Depletion

It is well known that platelets contribute a lot to hemostasis. Apart from its role in thrombus formation, platelets are highly involved in tumor progression and metastasis. In addition, it could also support tumor vascular homeostasis and protect the

integrity of tumor vessels (Kisucka et al., 2006; Ho-Tin-Noé et al., 2009). Studies showed that platelets reduction induced bleeding in the tumor site and increased leakiness of tumor vasculature. Therefore, platelets reduction in thrombocytopenic mice increased efficacy of chemotherapy for breast cancer (Demers et al., 2011). To avoid potential bleeding in normal organs caused by low platelet counts, a recent study by Li et al. designed a tumor microenvironment-responsive nanoparticle capable of delivering antiplatelet antibody R300 to selectively deplete platelet in tumor tissues, therefore augmenting vascular permeability and improving nanomedicine delivery for tumors (Li et al., 2017). Platelet depletion represented as a promising approach to augment transvascular delivery of nanomedicine to tumors.

Physical Stimulus

Radiation can improve tumor-targeted delivery of nanomedicine (Davies Cde et al., 2004; Giustini et al., 2012). Some possible mechanisms are as follows: First, radiation could upregulate the level of vascular endothelial growth factor (VEGF) by activating hypoxia inducible factor 1 (HIF1) (Moeller et al., 2004; Stapleton et al., 2016) or via multiple mitogen-activated protein kinase dependent pathways (Park et al., 2001) to increase the permeability of tumor vessels (Kobayashi et al., 2004). Results showed that the tumor vessels' permeability of magnetic resonance imaging-contrast agent with the molecular weight above 200 kDa was increased by 32.8% after irradiation (10 Gy). In addition, radiation can rapidly kill the sensitive tumor cells. The reduced cell density helped to alleviate compression stress from tumor cells, reopen collapsed vessels, and therefore increase tumor blood flow (Nagano et al., 2008; Khawar et al., 2015). The effect of radiation on tumors is complex and dose-, time-, and tumor-type dependent (Garcia-Barros et al., 2003; Fuks and Kolesnick, 2005; Kioi et al., 2010). Milosevic's recent review provides further evidence of the same (Stapleton et al., 2016).

Kong et al. pioneered the use of mild hyperthermia (HT) for nanomedicine extravasation into tumor tissues by improved vascular permeability (Kong et al., 2000, 2001). Studies further demonstrated that mild HT could also help improve tumor perfusion and reduce IFP (Sen et al., 2011; Winslow et al., 2015), probably via creation of vascular fenestrations and perturbation of the vascular endothelium (Kirui et al., 2015), and thus allow deep nanomedicine penetration throughout the tumors, rather than perivascular accumulation (Li et al., 2013). However, there was no direct evidence to demonstrate the pore size change of tumor vessels after HT treatment. The extravasation depth and intensity in tumor interstitium was considered to vary greatly among different types of tumors, which depends on the pattern of the endothelial lining and the intrinsic property of the surrounding tumor microenvironment (Eberhard et al., 2000), such as structure of the interstitial matrix. Apart from tumors with a certain vascular component, highly desmoplastic tumors could also respond well to mild HT treatment (Kirui et al., 2014, 2015). The temperature is a crucial element in the heating method, wherein results showed that 41–43°C was appropriate, because a very high temperature might damage the endothelial lining of tumor vessels and induce coagulation response. Thrombin formation could choke the vessels and compromise nanomedicine delivery. Alternatively, insufficient temperature might exert a very minimal effect on the tumor vessel endothelium to increase the endothelial gap (von Maltzahn et al., 2011; Li et al., 2013).

Ultrasound has been used to improve nanomedicine delivery for tumors by both mechanical and HT effect (Goins et al., 2016). For mechanical effects, many reports showed that gas-filled bubbles could be used to transiently produce pores in blood vessels (Durymanov et al., 2015) or cell membranes (sonoporation) (Yoon et al., 2014; Ma et al., 2016), through which nanomedicines of different types can effectively extravasate tumor vessels or enter into tumor cells (Thakkar et al., 2013), therefore achieving improved delivery of nanomedicine (Rapoport et al., 2013, 2015). Besides, ultrasound also produces heat at an acoustic intensity and in a time-dependent manner. Recently, Frazier used magnetic resonance imaging-guided, high-intensity focused ultrasound (HIFU) to produce a spatially uniform 43°C heating pattern in a xenograft tumor model and improved the accumulation of Evans blue dye in heated tumors to nearly 2-fold higher than in unheated tumors (Frazier et al., 2016).

Improving Interstitial Transport of Nanomedicine

ECM Disruption Strategies

Dense ECM always resists free penetration of nanomedicine throughout tumor tissues to reach tumor cells. Therefore, modification of ECM has been extensively explored to improve the delivery and distribution of nanomedicine in tumor tissues. The ECM modification strategy includes direct ECM disruption and reduction of ECM synthesis by inhibiting TAF activity.

Several studies have shown that different kinds of enzymes directly degrade the components of ECM such as collagen and hyaluronic acid and can improve the delivery of nanomedicine (**Table 1**). For instance, collagenase-coated nanomedicine could penetrate deeper into the core of *in vitro* tumor spheroids than control ones (Goodman et al., 2007; Cui et al., 2013). Besides, enzymatic digestion of collagen and decorin facilitates >10-fold increase in the diffusion of macromolecular dextran into tumor tissue, supporting matrix degradation as a useful tool to improve macromolecule distribution (Magzoub et al., 2008). In another study, intravenous injection of collagenase-1 into xenograft-bearing mice models increased the accumulation and gene expression of lipoplex in tumors by 1.5- and 2-fold, respectively, further confirming collagen digestion to be a useful strategy to improve nanomedicine delivery (Kato et al., 2012). Hyaluronan, or hyaluronic acid, a large linear glycosaminoglycan, composed of repeating N-acetyl glucosamine and glucuronic acid units, was also a crucial component of ECM (Provenzano et al., 2012), which was found abundant in non-small cell lung cancer (NSCLC), prostate, pancreatic, and breast cancers. Hyaluronidase was shown to induce a 4-fold increase in the distribution of liposomal doxorubicin in a human osteosarcoma xenograft model (Eikenes et al., 2005). A phase 1b trial of docetaxel combining PEGPH20 in metastatic

refractory NSCLC has been completed (NCT02346370) with results pending (Wong et al., 2017). In addition, a nanomedicine combining PEGylated hyaluronidase (PEGPH20) to improve the efficiency of chemotherapeutics for hyaluronan-high pancreatic cancer is currently in phase 3 clinical trial (NCT02715804) (Provenzano et al., 2012; Wong et al., 2017). Lysyl oxidase (LOX) is a key element in the crosslinking of collagen and increasing the stiffness of collagen fibers (Egeblad et al., 2010; Kanapathipillai et al., 2012). LOX-activity inhibition has proven successful in preventing ECM remodeling and stiffening (Levental et al., 2009; Barry-Hamilton et al., 2010), which may overcome the deregulated ECM barrier for nanomedicine delivery (Khawar et al., 2015).

It was noteworthy that ECM disruption was seldom used to increase nanomedicine delivery in highly vascularized tumors, which might be the relative lack of ECM in these tumors. However, our group found that fibrin, a kind of ECM component was rich in tumors harboring rich tumor vessels (Dvorak, 1986). The reason might be due to leakage of coagulation factors from circulation to tumor tissues and the high express level of tissue factor on tumor cells, both of which together contribute to local coagulation response in tumor tissues (Dvorak et al., 1985; Liu et al., 2011). As the end product of coagulation response, fibrin is mostly covalently cross-linked in tumor interstitium as an important component of tumor ECM (Dvorak, 1986; Pilch et al., 2006) and is mainly located in the vicinity tumor vessels (Nakahara et al., 2006), a distinct distribution pattern totally different from that of other components of matrix such as collagen and hyaluronic acid, which are always extensively distributed throughout tumor tissues. The special distribution pattern of fibrin was demonstrated to compress tumor vessels nearby, which reduce blood flow and compromise nanomedicine delivery for tumors. Moreover, as fibrin is always covalently cross-linked near tumor vessels, the penetration of nanomedicines in the tumor interstitium could also be hindered. Treatment with rtPA, a clinically widely used drug, at a dose of 25 mg/kg for 2 weeks, could safely and successfully deplete fibrin deposition, reopen compressed tumor vessels, reduce erythrocytes retention in tumor vessels, improve tumor blood flow, and further enhance the accumulation and penetration of nanoparticles (**Figure 3**; Zhang et al., 2016b).

However, systematic treatment with ECM disruptors such as collagenase, hyaluronidase, and rtPA may cause damage to healthy tissues, and site-specific action might be safer for clinical transformation. Therefore, tumor-specific degradation of ECM was achieved by coating nanomedicines with specific ECM enzymes (Zhou et al., 2016) or PEGlated ECM enzymes (Hingorani et al., 2016). Zhou et al. showed that hyaluronidase modified on the surface of nanoparticles was more effective than free hyaluronidase to help facilitate nanoparticle diffusion and achieved better therapeutic benefits (Zhou et al., 2016). Reports also showed that the dose of hyaluronidase seemed critical, because high doses of hyaluronidase may collapse water-swelling cage structures of hyaluronan rendering the ECM more viscous and less permeable, thereby reducing the diffusion coefficient of nanomedicine (Eikenes et al., 2010). In another research, Bromelain, a crude enzymatic complex purified from pineapple stems that belongs to the peptidase papain family, was decorated to mesoporous silica nanoparticles (Br-MSN), which showed an increased ability to digest and diffuse in tumor ECM *in vitro* and *in vivo* (Parodi et al., 2014).

TAF was mainly responsible for ECM production. Resting fibroblasts were transformed to TAF by cancer-derived growth factors such as TGF-β, Hedgehog moiety (Olive et al., 2009; Stylianopoulos et al., 2012), and PDGF (Olson and Hanahan, 2009). This trans-differentiation process of TAF is always characterized by the encoding of ECM-associated components such as collagens, hyaluronan, fibronectin, and MMPs (Cirri and Chiarugi, 2011). Therefore, ECM deregulation can be realized by blocking the growth factors involved in signaling for TAF stimulation. Taking TGF-β-associated signaling as an example, antibodies or other agents capable of blocking TGF-β signaling have proven to inhibit collagen synthesis and enhance nanomedicine delivery in xenograft models. The TGF-β neutralizing antibody ID11 improved tumor tissue delivery of Doxil and obtained better control of mammary carcinoma in xenograft models (Liu et al., 2012). Recently, our group used cyclopamine, a naturally occurring steroidal alkaloid, to inhibit the Hedgehog signaling pathway which contributes a lot to ECM production in pancreatic carcinoma by acting on the Smoothened (SMO) receptor (Heretsch et al., 2010). Compared with the control group, cyclopamine treatment successfully disrupt ECM in in pancreatic cancer, increased functional vessels about 2 folds at a dose of 50 mg/kg for 3 weeks, and significantly improved the accumulation (by a 2.6-fold) and penetration of nanoparticles in tumor tissues (Zhang et al., 2016c).

ECM modulation suits nanomedicine regardless of their size, because ECM modification could reopen compressed vasculatures to improve tumor blood flow and decrease the hindrance of nanomedicine penetrating ECM to reach tumor cells (Chauhan and Jain, 2013). Generally speaking, this strategy most benefits the delivery of larger nanomedicines (Cabral et al., 2011; Jacobetz et al., 2012), as they are more hindered by the ECM (Pluen et al., 2001).

Stromal Cell Reprogramming or Depletion Strategies

Apart from dense ECM, desmoplastic tumors always harbor a high density of stromal cells, among which TAF has been regarded as the major component of tumoral stroma as a potential therapeutic target for nanomedicine delivery. TAF depletion can improve the interstitial transport and distribution of nanomedicine by optimization of the tumor interstitium. Quercetin nanoparticles, capable of suppressing Wnt16 expression could reduce the number of TAF and improve nanomedicine delivery to bladder carcinoma (Hu et al., 2017). Inspired by the close association between cyclooxygenase-2 (COX-2) and tumor-associated angiogenesis, as well as tumor matrix formation, our group explored the tumor microenvironment modulation effect of celecoxib, a special COX-2 inhibitor widely used in clinics. Very interestingly, oral celecoxib treatment at a dose of 200 mg/kg for 2 weeks could successfully normalized the tumor microenvironment, including tumor-associated fibroblast depletion, fibronectin bundle disruption, tumor vessel normalization, and tumor

perfusion improvement. Furthermore, it also significantly enhanced the *in vivo* accumulation and deep penetration of 22-nm micelles rather than 100-nm nanoparticles in tumor tissues and improved the therapeutic efficacy of paclitaxel-loaded micelles in tumor xenograft-bearing mouse models (Zhang et al., 2017b).

Although TAF depletion could undoubtedly modify the tumor microenvironment to improve nanomedicine delivery, recent studies have also indicated that direct TAF depletion might drive tumor metastasis and progression (Özdemir et al., 2014), suggesting a paradoxical effect of TAF depletion. One explanation for this paradoxical effect is that the TAF-depleting strategy runs the risk of eliminating the key element needed for tissue homeostasis (Miao et al., 2015). To avoid this paradox, an alternative approach is to transform activated TAF to a dormant form. Losartan is a antihypertensive agent with anti-fibrosis properties. Research has shown that losartan treatment reprogramed TAF and reduced the collagen and hyaluronan content in desmoplastic models of human breast and pancreatic tumors in mice and improved the distribution and therapeutic effects of systematic administered Doxil (Diop-Frimpong et al., 2011; Chauhan et al., 2013). The antifibrotic effect of losartan was associated with reduced number of activated TAFs and therefore decreased expression of downstream profibrotic factors, such as connective tissue growth factor (CTGF), TGF-β1, and ET-1 (Chauhan et al., 2013), which led to an ongoing clinical trial of losartan combined with chemotherapy in pancreatic tumors (NCT01821729). Vitamin D receptor (VDR) and Wnt-β-Catenin signaling pathway was upregulated in pancreatic stellate cell (PSC), a form of TAF in PDA (Omary et al., 2007). Other studies showed that the VDR ligand and all-trans retinoic acid (ATRA) can act through VDR or Wnt-β-Catenin signaling pathway to reprogram PSC to the quiescent state to reduce the fibrotic content in tumor interstitium (Froeling et al., 2011; Sherman et al., 2014; Chronopoulos et al., 2016), which is promising for the second-wave nanomedicine therapy.

TAMs are prominent components and critical modulators of the tumor microenvironment and contribute to tumor development, invasion, and metastases. Evidences have shown TAMs protect tumor cells from chemotherapy and suppress the immune response of cytotoxic T cells (Jinushi et al., 2011), highlighting the essential of targeting TAMs for cancer treatment. TAM depletion could decrease off-target uptake of nanomedicine by TAMs and thus increase drug delivery to tumor cells. However, the effect of selective TAM depletion on nanomedicine delivery for tumor was seldom reported, and TAM depletion might be a new strategy for tumor microenvironment modulation to enhance tumor nanomedicine delivery.

FUTURE PERSPECTIVES

Nanomedicine drug delivery system has attracted extensive attention in the field of tumor treatment. The complex tumor microenvironment including structural abnormalities in tumor vessels, dense ECM structure, and high density of stromal cells as well as physicochemical environment such as elevated IFP pose barriers and compromise the delivery of nanomedicines**.** In this review, we summarized these barriers and provided strategies to overcome them for improved nanomedicine delivery.

However, some aspects deserve special attention. First, tumors could be generally divided into two types such as those with abundant permeable but uncompressed vessels and tumors with dense ECM and large amount of TAF. More precise classification of tumor microenvironment-like tumor cells might be urgently needed for precise tumor nanomedicine delivery. We need to adopt different strategies according to the characteristics of the tumor microenvironment. For example, tumor vessel normalization is more effective for tumors with abundant, highly permeable but not compressed vessels, but not so appropriate for highly desmoplastic tumors. ECM disruption strategy demonstrates promising prospects to enhance nanomedicine delivery for tumors with abundant ECM even in clinical trials. As for tumors with a certain amount of both vessels and ECM, strategies capable of modulating vessels and ECM should be combined to obtain an optimal effect. In addition, it is important to study new animal models capable of quantitative analysis of parameters involved in the tumor microenvironment, such as IFP to quantify the negative contribution of tumor microenvironment parameters to tumor nanomedicine delivery and help develop corresponding strategies. Furthermore, the tumor microenvironment is too complex and one strategy might have multiple modulation effects on the tumor microenvironment. With respect to ECM disruption, it not only disrupt tumor ECM to optimize interstitial transport of nanomedicines, but also alleviate compression for tumor vessels to improve tumor perfusion to bring more nanomedicines to the tumor site. Another good illustration is the tumor vessel normalization strategy. Tumor vessel normalization could repair tumor vessels structure, which could improve tumor perfusion and reduce IFP to increase the delivery of small nanomedicine around 20–40 nm. However, it inversely compromised the delivery of larger nanomedicine around 100 nm because of the size reduction of endothelial gaps.

Therefore, nanomedicines with suitable qualities should be combined with approaches modulating the tumor microenvironment to overcome nanomedicine transport barriers that the advanced design of nanomedicines cannot conquer. With these in mind, we believe nanomedicines of the future could be far more effective than those available at present.

AUTHOR CONTRIBUTIONS

ZP and YH conceived the principal idea and revised the manuscripts. BZ and ZP co-wrote the manuscript.

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REFERENCES

Allen, T. M., and Cullis, P. R. (2004). Drug delivery systems: entering the mainstream. *Science* 303, 1818–1822. doi: 10.1126/science.1095833

Atsumi, T., Singh, R., Sabharwal, L., Bando, H., Meng, J., Arima, Y., et al. (2014). Inflammation amplifier, a new paradigm in cancer biology. *Cancer Res.* 74, 8–14. doi: 10.1158/0008-5472.CAN-13-2322

Bailey, J. M., Swanson, B. J., Hamada, T., Eggers, J. P., Singh, P. K., Caffery, T., et al. (2008). Sonic hedgehog promotes desmoplasia in pancreatic cancer. *Clin. Cancer Res.* 14, 5995–6004. doi: 10.1158/1078-0432.CCR-08-0291

Barry-Hamilton, V., Spangler, R., Marshall, D., McCauley, S., Rodriguez, H. M., Oyasu, M., et al. (2010). Allosteric inhibition of lysyl oxidase-like-2 impedes the development of a pathologic microenvironment. *Nat. Med.* 16, 1009–1017. doi: 10.1038/nm.2208

Boucher, Y., Baxter, L. T., and Jain, R. K. (1990). Interstitial pressure gradients in tissue-isolated and subcutaneous tumors: implications for therapy. *Cancer Res.* 50, 4478–4484.

Brennen, W. N., Rosen, D. M., Wang, H., Isaacs, J. T., and Denmeade, S. R. (2012). Targeting carcinoma-associated fibroblasts within the tumor stroma with a fibroblast activation protein-activated prodrug. *J. Natl. Cancer Inst.* 104, 1320–1334. doi: 10.1093/jnci/djs336

Cabral, H., Matsumoto, Y., Mizuno, K., Chen, Q., Murakami, M., Kimura, M., et al. (2011). Accumulation of sub-100 nm polymeric micelles in poorly permeable tumours depends on size. *Nat. Nanotechnol.* 6, 815–823. doi: 10.1038/nnano.2011.166

Carmeliet, P., and Jain, R. K. (2000). Angiogenesis in cancer and other diseases. *Nature* 407, 249–257. doi: 10.1038/35025220

Carmeliet, P., and Jain, R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473, 298–307. doi: 10.1038/nature10144

Chakroborty, D., Sarkar, C., Yu, H., Wang, J., Liu, Z., Dasgupta, P. S., et al. (2011). Dopamine stabilizes tumor blood vessels by up-regulating angiopoietin 1 expression in pericytes and Kruppel-like factor-2 expression in tumor endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20730–20735. doi: 10.1073/pnas.1108696108

Chary, S. R., and Jain, R. K. (1989). Direct measurement of interstitial convection and diffusion of albumin in normal and neoplastic tissues by fluorescence photobleaching. *Proc. Natl. Acad. Sci. U.S.A.* 86, 5385–5389. doi: 10.1073/pnas.86.14.5385

Chauhan, V. P., and Jain, R. K. (2013). Strategies for advancing cancer nanomedicine. *Nat. Mater.* 12, 958–962. doi: 10.1038/nmat3792

Chauhan, V. P., Lanning, R. M., Diop-Frimpong, B., Mok, W., Brown, E. B., Padera, T. P., et al. (2009). Multiscale measurements distinguish cellular and interstitial hindrances to diffusion *in vivo*. *Biophys. J.* 97, 330–336. doi: 10.1016/j.bpj.2009.03.064

Chauhan, V. P., Martin, J. D., Liu, H., Lacorre, D. A., Jain, S. R., Kozin, S. V., et al. (2013). Angiotensin inhibition enhances drug delivery and potentiates chemotherapy by decompressing tumour blood vessels. *Nat. Commun.* 4:2516. doi: 10.1038/ncomms3516

Chauhan, V. P., Popović, Z., Chen, O., Cui, J., Fukumura, D., Bawendi, M. G., et al. (2011). Fluorescent nanorods and nanospheres for real-time *in vivo* probing of nanoparticle shape-dependent tumor penetration. *Angew. Chem. Int. Ed.* 50, 11417–11420. doi: 10.1002/anie.201104449

Chauhan, V. P., Stylianopoulos, T., Martin, J. D., Popovic, Z., Chen, O., Kamoun, W. S., et al. (2012). Normalization of tumour blood vessels improves the delivery of nanomedicines in a size-dependent manner. *Nat. Nanotechnol.* 7, 383–388. doi: 10.1038/nnano.2012.45

Chronopoulos, A., Robinson, B., Sarper, M., Cortes, E., Auernheimer, V., Lachowski, D., et al. (2016). ATRA mechanically reprograms pancreatic stellate cells to suppress matrix remodelling and inhibit cancer cell invasion. *Nat. Commun.* 7:12630. doi: 10.1038/ncomms12630

Cieslewicz, M., Tang, J., Yu, J. L., Cao, H., Zavaljevski, M., Motoyama, K., et al. (2013). Targeted delivery of proapoptotic peptides to tumor-associated macrophages improves survival. *Proc. Natl. Acad. Sci. U.S.A.* 110, 15919–15924. doi: 10.1073/pnas.1312197110

Cirri, P., and Chiarugi, P. (2011). Cancer associated fibroblasts: the dark side of the coin. *Am. J. Cancer Res.* 1, 482–497.

Cui, M., Naczynski, D. J., Zevon, M., Griffith, C. K., Sheihet, L., Poventud-Fuentes, I., et al. (2013). Multifunctional albumin nanoparticles as combination drug carriers for intra-tumoral chemotherapy. *Adv. Healthc. Mater.* 2, 1236–1245. doi: 10.1002/adhm.201200467

Curnis, F., Sacchi, A., and Corti, A. (2002). Improving chemotherapeutic drug penetration in tumors by vascular targeting and barrier alteration. *J. Clin. Invest.* 110, 475–482. doi: 10.1172/JCI0215223

Davies Cde, L., Lundstrøm, L. M., Frengen, J., Eikenes, L., Bruland, S. O., Kaalhus, O., et al. (2004). Radiation improves the distribution and uptake of liposomal doxorubicin (caelyx) in human osteosarcoma xenografts. *Cancer Res.* 64, 547–553. doi: 10.1158/0008-5472.CAN-03-0576

Demers, M., Ho-Tin-Noé, B., Schatzberg, D., Yang, J. J., and Wagner, D. D. (2011). Increased efficacy of breast cancer chemotherapy in thrombocytopenic mice. *Cancer Res.* 71, 1540–1549. doi: 10.1158/0008-5472.CAN-10-2038

Dequidt, C., Danglot, L., Alberts, P., Galli, T., Choquet, D., and Thoumine, O. (2007). Fast turnover of L1 adhesions in neuronal growth cones involving both surface diffusion and exo/endocytosis of L1 molecules. *Mol. Biol. Cell* 18, 3131–3143. doi: 10.1091/mbc.E06-12-1101

Diop-Frimpong, B., Chauhan, V. P., Krane, S., Boucher, Y., and Jain, R. K. (2011). Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors. *Proc. Natl. Acad. Sci. U.S.A.* 108, 2909–2914. doi: 10.1073/pnas.1018892108

Dreher, M. R., Liu, W., Michelich, C. R., Dewhirst, M. W., Yuan, F., and Chilkoti, A. (2006). Tumor vascular permeability, accumulation, and penetration of macromolecular drug carriers. *J. Natl. Cancer Inst.* 98, 335–344. doi: 10.1093/jnci/djj070

Durymanov, M. O., Rosenkranz, A. A., and Sobolev, A. S. (2015). Current approaches for improving intratumoral accumulation and distribution of nanomedicines. *Theranostics* 5, 1007–1020. doi: 10.7150/thno.11742

Dvorak, H. F. (1986). Tumors: wounds that do not heal: similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* 315, 1650–1659.

Dvorak, H. F., Senger, D. R., Dvorak, A. M., Harvey, V. S., and McDonagh, J. (1985). Regulation of extravascular coagulation by microvascular permeability. *Science* 227, 1059–1061. doi: 10.1126/science.3975602

Eberhard, A., Kahlert, S., Goede, V., Hemmerlein, B., Plate, K. H., and Augustin, H. G. (2000). Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. *Cancer Res.* 60, 1388–1393.

Egeblad, M., Rasch, M. G., and Weaver, V. M. (2010). Dynamic interplay between the collagen scaffold and tumor evolution. *Curr. Opin. Cell Biol.* 22, 697–706. doi: 10.1016/j.ceb.2010.08.015

Eikenes, L., Tari, M., Tufto, I., Bruland, O. S., and de Lange Davies, C. (2005). Hyaluronidase induces a transcapillary pressure gradient and improves the distribution and uptake of liposomal doxorubicin (Caelyx) in human osteosarcoma xenografts. *Br. J. Cancer* 93, 81–88. doi: 10.1038/sj.bjc.6602626

Eikenes, L., Tufto, I., Schnell, E. A., Bjørkøy, A., and De Lange Davies, C. (2010). Effect of collagenase and hyaluronidase on free and anomalous diffusion in multicellular spheroids and xenografts. *Anticancer Res.* 30, 359–368.

Ellis, L. M. (2005). Bevacizumab. *Nat. Rev. Drug Discov.* 3, 995–996. doi: 10.1038/nrd1727

Fang, J., Nakamura, H., and Maeda, H. (2011). The EPR effect: unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. *Adv. Drug Delivery Rev.* 63, 136–151. doi: 10.1016/j.addr.2010.04.009

Feig, C., Gopinathan, A., Neesse, A., Chan, D. S., Cook, N., and Tuveson, D. A. (2012). The pancreas cancer microenvironment. *Clin. Cancer Res.* 18, 4266–4276. doi: 10.1158/1078-0432.CCR-11-3114

Frantz, C., Stewart, K. M., and Weaver, V. M. (2010). The extracellular matrix at a glance. *J. Cell Sci.* 123, 4195–4200. doi: 10.1242/jcs.023820

Frazier, N., Payne, A., de Bever, J., Dillon, C., Panda, A., Subrahmanyam, N., et al. (2016). High intensity focused ultrasound hyperthermia for enhanced macromolecular delivery. *J. Control. Release* 241, 186–193. doi: 10.1016/j.jconrel.2016.09.030

Froeling, F. E., Feig, C., Chelala, C., Dobson, R., Mein, C. E., Tuveson, D. A., et al. (2011). Retinoic acid-induced pancreatic stellate cell quiescence reduces paracrine Wnt-beta-catenin signaling to slow tumor progression. *Gastroenterology* 141, 486–497. doi: 10.1053/j.gastro.2011.06.047

Fuks, Z., and Kolesnick, R. (2005). Engaging the vascular component of the tumor response. *Cancer Cell* 8, 89–91. doi: 10.1016/j.ccr.2005.07.014

Garcia-Barros, M., Paris, F., Cordon-Cardo, C., Lyden, D., Rafii, S., Haimovitz-Friedman, A., et al. (2003). Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science* 300, 1155–1159. doi: 10.1126/science.1082504

Giustini, A. J., Petryk, A. A., and Hoopes, P. J. (2012). Ionizing radiation increases systemic nanoparticle tumor accumulation. *Nanomedicine* 8, 818–821. doi: 10.1016/j.nano.2012.05.001

Goel, S., Duda, D. G., Xu, L., Munn, L. L., Boucher, Y., Fukumura, D., et al. (2011). Normalization of the vasculature for treatment of cancer and other diseases. *Physiol. Rev.* 91, 1071–1121. doi: 10.1152/physrev.00038.2010

Goins, B., Phillips, W. T., and Bao, A. (2016). Strategies for improving the intratumoral distribution of liposomal drugs in cancer therapy. *Expert Opin. Drug Deliv.* 13, 873–889. doi: 10.1517/17425247.2016.1167035

Gong, H., Chao, Y., Xiang, J., Han, X., Song, G., Feng, L., et al. (2016). Hyaluronidase To Enhance Nanoparticle-Based Photodynamic Tumor Therapy. *Nano Lett.* 16, 2512–2521. doi: 10.1021/acs.nanolett.6b00068

Goodman, T. T., Olive, P. L., and Pun, S. H. (2007). Increased nanoparticle penetration in collagenase-treated multicellular spheroids. *Int. J. Nanomedicine* 2, 265–274.

Gradishar, W. J., Tjulandin, S., Davidson, N., Shaw, H., Desai, N., Bhar, P., et al. (2005). Phase III trial of nanoparticle albumin-bound paclitaxel compared with polyethylated castor oil-based paclitaxel in women with breast cancer. *J. Clin. Oncol.* 23, 7794–7803. doi: 10.1200/JCO.2005.04.937

Guo, S., Lin, C. M., Xu, Z., Miao, L., Wang, Y., and Huang, L. (2014). Co-Delivery of Cisplatin and Rapamycin For Enhanced Anticancer Therapy Through Synergistic Effects and Microenvironment Modulation. *ACS Nano* 8, 4996–5009. doi: 10.1021/nn5010815

Han, S. S., Li, Z. Y., Zhu, J. Y., Han, K., Zeng, Z. Y., Hong, W., et al. (2015). Dual-pH Sensitive Charge-Reversal Polypeptide Micelles for Tumor-Triggered Targeting Uptake and Nuclear Drug Delivery. *Small* 11, 2543–2554. doi: 10.1002/smll.201402865

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Heretsch, P., Tzagkaroulaki, L., and Giannis, A. (2010). Cyclopamine and hedgehog signaling: chemistry, biology, medical perspectives. *Angew. Chem., Int. Ed.* 49, 3418–3427. doi: 10.1002/anie.200906967

Hingorani, S. R., Harris, W. P., Beck, J. T., Berdov, B. A., Wagner, S. A., Pshevlotsky, E. M., et al. (2016). Phase Ib Study of PEGylated Recombinant Human Hyaluronidase and Gemcitabine in Patients with Advanced Pancreatic Cancer. *Clin. Cancer Res.* 22, 2848–2854. doi: 10.1158/1078-0432.CCR-15-2010

Ho-Tin-Noé, B., Goerge, T., and Wagner, D. D. (2009). Platelets: guardians of tumor vasculature. *Cancer Res.* 69, 5623–5626. doi: 10.1158/0008-5472.CAN-09-1370

Hu, K., Miao, L., Goodwin, T. J., Li, J., Liu, Q., and Huang, L. (2017). Quercetin remodels the tumor microenvironment to improve the permeation, retention, and antitumor effects of nanoparticles. *ACS Nano* 11, 4916–4925. doi: 10.1021/acsnano.7b01522

Hu, W., Li, X., Zhang, C., Yang, Y., Jiang, J., and Wu, C. (2016). Tumor-associated macrophages in cancers. *Clin. Transl. Oncol.* 18, 251–258. doi: 10.1007/s12094-015-1373-0

Huang, S., Shao, K., Liu, Y., Kuang, Y., Li, J., An, S., et al. (2013). Tumor-targeting and microenvironment-responsive smart nanoparticles for combination therapy of antiangiogenesis and apoptosis. *ACS Nano* 7, 2860–2871. doi: 10.1021/nn400548g

Huang, Y., Yuan, J., Righi, E., Kamoun, W. S., Ancukiewicz, M., Nezivar, J., et al. (2012). Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy. *Proc. Natl. Acad. Sci. U.S.A.* 109, 17561–17566. doi: 10.1073/pnas.1215397109

Jacobetz, M. A., Chan, D. S., Neesse, A., Bapiro, T. E., Cook, N., Frese, K. K., et al. (2012). Hyaluronan impairs vascular function and drug delivery in a mouse model of pancreatic cancer. *Gut* 62, 112–120. doi: 10.1136/gutjnl-2012-302529

Jain, R. K. (1987). Transport of molecules in the tumor interstitium: a review. *Cancer Res.* 47, 3039–3051.

Jain, R. K. (1994). Barriers to drug delivery in solid tumors. *Sci. Am.* 271, 58–65. doi: 10.1038/scientificamerican0794-58

Jain, R. K. (2003). Molecular regulation of vessel maturation. *Nat. Med.* 9, 685–693. doi: 10.1038/nm0603-685

Jain, R. K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 307, 58–62. doi: 10.1126/science.1104819

Jain, R. K. (2013). Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers. *J. Clin. Oncol.* 31, 2205–2218. doi: 10.1200/JCO.2012.46.3653

Jain, R. K., and Stylianopoulos, T. (2010). Delivering nanomedicine to solid tumors. *Nat. Rev. Clin. Oncol.* 7, 653–664. doi: 10.1038/nrclinonc.2010.139

Jain, R. K., Martin, J. D., and Stylianopoulos, T. (2014). The role of mechanical forces in tumor growth and therapy. *Annu. Rev. Biomed. Eng.* 16, 321–346. doi: 10.1146/annurev-bioeng-071813-105259

Järveläinen, H., Sainio, A., Koulu, M., Wight, T. N., and Penttinen, R. (2009). Extracellular matrix molecules: potential targets in pharmacotherapy. *Pharmacol. Rev.* 61, 198–223. doi: 10.1124/pr.109.001289

Jiang, T., Zhang, B., Shen, S., Tuo, Y., Luo, Z., Hu, Y., et al. (2017). Tumor microenvironment modulation by cyclopamine improved photothermal therapy of biomimetic gold nanorods for pancreatic ductal adenocarcinomas. *ACS Appl. Mater. Interfaces* 9, 31497–31508. doi: 10.1021/acsami.7b09458

Jinushi, M., Chiba, S., Yoshiyama, H., Masutomi, K., Kinoshita, I., Dosaka-Akita, H., et al. (2011). Tumor-associated macrophages regulate tumorigenicity and anticancer drug responses of cancer stem/initiating cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12425–12430. doi: 10.1073/pnas.1106645108

Kanapathipillai, M., Mammoto, A., Mammoto, T., Kang, J. H., Jiang, E., Ghosh, K., et al. (2012). Inhibition of mammary tumor growth using lysyl oxidase-targeting nanoparticles to modify extracellular matrix. *Nano Lett.* 12, 3213–3217. doi: 10.1021/nl301206p

Kano, M. R., Bae, Y., Iwata, C., Morishita, Y., Yashiro, M., Oka, M., et al. (2007). Improvement of cancer-targeting therapy, using nanocarriers for intractable solid tumors by inhibition of TGF-beta signaling. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3460–3465. doi: 10.1073/pnas.0611660104

Kato, M., Hattori, Y., Kubo, M., and Maitani, Y. (2012). Collagenase-1 injection improved tumor distribution and gene expression of cationic lipoplex. *Int. J. Pharm.* 423, 428–434. doi: 10.1016/j.ijpharm.2011.12.015

Khawar, I. A., Kim, J. H., and Kuh, H. J. (2015). Improving drug delivery to solid tumors: priming the tumor microenvironment. *J. Control. Release* 201, 78–89. doi: 10.1016/j.jconrel.2014.12.018

Kioi, M., Vogel, H., Schultz, G., Hoffman, R. M., Harsh, G. R., and Brown, J. M. (2010). Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. *J. Clin. Invest.* 120, 694–705. doi: 10.1172/JCI40283

Kirtane, A. R., Sadhukha, T., Kim, H., Khanna, V., Koniar, B., and Panyam, J. (2017). Fibrinolytic Enzyme Cotherapy Improves Tumor Perfusion and Therapeutic Efficacy of Anticancer Nanomedicine. *Cancer Res.* 77, 1465–1475. doi: 10.1158/0008-5472.CAN-16-1646

Kirui, D. K., Celia, C., Molinaro, R., Bansal, S. S., Cosco, D., Fresta, M., et al. (2015). Mild hyperthermia enhances transport of liposomal gemcitabine and improves *in vivo* therapeutic response. *Adv. Healthc. Mater.* 4, 1092–1103. doi: 10.1002/adhm.201400738

Kirui, D. K., Koay, E. J., Guo, X., Cristini, V., Shen, H., and Ferrari, M. (2014). Tumor vascular permeabilization using localized mild hyperthermia to improve macromolecule transport. *Nanomedicine* 10, 1487–1496. doi: 10.1016/j.nano.2013.11.001

Kisucka, J., Butterfield, C. E., Duda, D. G., Eichenberger, S. C., Saffaripour, S., Ware, J., et al. (2006). Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage. *Proc. Natl. Acad. Sci. U.S.A.* 103, 855–860. doi: 10.1073/pnas.0510412103

Kobayashi, H., Reijnders, K., English, S., Yordanov, A. T., Milenic, D. E., Sowers, A. L., et al. (2004). Application of a macromolecular contrast agent for detection of alterations of tumor vessel permeability induced by radiation. *Clin. Cancer Res.* 10, 7712–7720. doi: 10.1158/1078-0432.CCR-04-1175

Kong, G., Braun, R. D., and Dewhirst, M. W. (2000). Hyperthermia enables tumor-specific nanoparticle delivery: effect of particle size. *Cancer Res.* 60, 4440–4445.

Kong, G., Braun, R. D., and Dewhirst, M. W. (2001). Characterization of the effect of hyperthermia on nanoparticle extravasation from tumor vasculature. *Cancer Res.* 61, 3027–3032.

Leu, A. J., Berk, D. A., Lymboussaki, A., Alitalo, K., and Jain, R. K. (2000). Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. *Cancer Res.* 60, 4324–4327.

Levental, K. R., Yu, H., Kass, L., Lakins, J. N., Egeblad, M., Erler, J. T., et al. (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* 139, 891–906. doi: 10.1016/j.cell.2009.10.027

Levick, J. R. (1987). Flow through interstitium and other fibrous matrices. *Q. J. Exp. Physiol.* 72, 409–437. doi: 10.1113/expphysiol.1987.sp003085

Li, L., ten Hagen, T. L., Bolkestein, M., Gasselhuber, A., Yatvin, J., van Rhoon, G. C., et al. (2013). Improved intratumoral nanoparticle extravasation and penetration by mild hyperthermia. *J. Controlled Release* 167, 130–137. doi: 10.1016/j.jconrel.2013.01.026

Li, Q., Hata, A., Kosugi, C., Kataoka, N., and Funaki, M. (2010). The density of extracellular matrix proteins regulates inflammation and insulin signaling in adipocytes. *FEBS Lett.* 584, 4145–4150. doi: 10.1016/j.febslet.2010.08.033

Li, S., Zhang, Y., Wang, J., Zhao, Y., Ji, T., Zhao, X., et al. (2017). Nanoparticle-mediated local depletion of tumour-associated platelets disrupts vascular barriers and augments drug accumulation in tumours. *Nat. Biomed. Eng.* 1, 667–679. doi: 10.1038/s41551-017-0115-8

Liu, J., Liao, S., Diop-Frimpong, B., Chen, W., Goel, S., Naxerova, K., et al. (2012). TGF-beta blockade improves the distribution and efficacy of therapeutics in breast carcinoma by normalizing the tumor stroma. *Proc. Natl. Acad. Sci. U.S.A.* 109, 16618–16623. doi: 10.1073/pnas.1117610109

Liu, Y., Jiang, P., Capkova, K., Xue, D., Ye, L., Sinha, S. C., et al. (2011). Tissue factor-activated coagulation cascade in the tumor microenvironment is critical for tumor progression and an effective target for therapy. *Cancer Res.* 71, 6492–6502. doi: 10.1158/0008-5472.CAN-11-1145

Lu, P., Weaver, V. M., and Werb, Z. (2012). The extracellular matrix: a dynamic niche in cancer progression. *J. Cell Biol.* 196, 395–406. doi: 10.1083/jcb.201102147

Ma, J., Shen, M., Xu, C. S., Sun, Y., Duan, Y. R., and Du, L. F. (2016). Biodegradable double-targeted PTX-mPEG-PLGA nanoparticles for ultrasound contrast enhanced imaging and antitumor therapy *in vitro*. *Oncotarget* 7, 80008–80018. doi: 10.18632/oncotarget.13243

Maeda, H. (2012). Macromolecular therapeutics in cancer treatment: the EPR effect and beyond. *J. Control. Release* 164, 138–144. doi: 10.1016/j.jconrel.2012.04.038

Maes, H., Kuchnio, A., Peric, A., Moens, S., Nys, K., De Bock, K., et al. (2014). Tumor vessel normalization by chloroquine independent of autophagy. *Cancer Cell* 26, 190–206. doi: 10.1016/j.ccr.2014.06.025

Magzoub, M., Jin, S., and Verkman, A. S. (2008). Enhanced macromolecule diffusion deep in tumors after enzymatic digestion of extracellular matrix collagen and its associated proteoglycan decorin. *FASEB J.* 22, 276–284. doi: 10.1096/fj.07-9150com

Martinive, P., De Wever, J., Bouzin, C., Baudelet, C., Sonveaux, P., Grégoire, V., et al. (2006). Reversal of temporal and spatial heterogeneities in tumor perfusion identifies the tumor vascular tone as a tunable variable to improve drug delivery. *Mol. Cancer Ther.* 5, 1620–1627. doi: 10.1158/1535-7163.MCT-05-0472

McKee, T. D., Grandi, P., Mok, W., Alexandrakis, G., Insin, N., Zimmer, J. P., et al. (2006). Degradation of fibrillar collagen in a human melanoma xenograft improves the efficacy of an oncolytic herpes simplex virus vector. *Cancer Res.* 66, 2509–2513. doi: 10.1158/0008-5472.CAN-05-2242

Meng, H., Zhao, Y., Dong, J., Xue, M., Lin, Y. S., Ji, Z., et al. (2013). Two-wave nanotherapy to target the stroma and optimize gemcitabine delivery to a human pancreatic cancer model in mice. *ACS Nano* 7, 10048–10065. doi: 10.1021/nn404083m

Miao, L., and Huang, L. (2015). Exploring the tumor microenvironment with nanoparticles. *Cancer Treat. Res.* 166, 193–226. doi: 10.1007/978-3-319-16555-4_9

Miao, L., Lin, C. M., and Huang, L. (2015). Stromal barriers and strategies for the delivery of nanomedicine to desmoplastic tumors. *J. Control. Release* 219, 192–204. doi: 10.1016/j.jconrel.2015.08.017

Miao, L., Newby, J. M., Lin, C. M., Zhang, L., Xu, F., Kim, W. Y., et al. (2016). The binding site barrier elicited by tumor-associated fibroblasts interferes disposition of nanoparticles in stroma-vessel type tumors. *ACS Nano* 10, 9243–9258. doi: 10.1021/acsnano.6b02776

Milosevic, M., Fyles, A., Hedley, D., and Hill, R. (2004). The human tumor microenvironment: invasive (needle) measurement of oxygen and interstitial fluid pressure. *Semin. Radiat. Oncol.* 14, 249–258. doi: 10.1016/j.semradonc.2004.04.006

Mocellin, S., Wang, E., and Marincola, F. M. (2001). Cytokines and immune response in the tumor microenvironment. *J. Immunother.* 24, 392–407. doi: 10.1097/00002371-200109000-00002

Moeller, B. J., Cao, Y., Li, C. Y., and Dewhirst, M. W. (2004). Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. *Cancer Cell* 5, 429–441. doi: 10.1016/S1535-6108(04)00115-1

Mok, W., Boucher, Y., and Jain, R. K. (2007). Matrix metalloproteinases-1 and−8 improve the distribution and efficacy of an oncolytic virus. *Cancer Res.* 67, 10664–10668. doi: 10.1158/0008-5472.CAN-07-3107

Monsky, W. L., Fukumura, D., Gohongi, T., Ancukiewcz, M., Weich, H. A., Torchilin, V. P., et al. (1999). Augmentation of transvascular transport of macromolecules and nanoparticles in tumors using vascular endothelial growth factor. *Cancer Res.* 59, 4129–4135.

Nagano, S., Perentes, J. Y., Jain, R. K., and Boucher, Y. (2008). Cancer cell death enhances the penetration and efficacy of oncolytic herpes simplex virus in tumors. *Cancer Res.* 68, 3795–3802. doi: 10.1158/0008-5472.CAN-07-6193

Nakahara, T., Norberg, S. M., Shalinsky, D. R., Hu-Lowe, D. D., and McDonald, D. M. (2006). Effect of inhibition of vascular endothelial growth factor signaling on distribution of extravasated antibodies in tumors. *Cancer Res.* 66, 1434–1445. doi: 10.1158/0008-5472.CAN-05-0923

Netti, P. A., Berk, D. A., Swartz, M. A., Grodzinsky, A. J., and Jain, R. K. (2000). Role of extracellular matrix assembly in interstitial transport in solid tumors. *Cancer Res.* 60, 2497–2503.

Netti, P. A., Roberge, S., Boucher, Y., Baxter, L. T., and Jain, R. K. (1996). Effect of transvascular fluid exchange on pressure-flow relationship in tumors: a proposed mechanism for tumor blood flow heterogeneity. *Microvasc. Res.* 52, 27–46. doi: 10.1006/mvre.1996.0041

Nichols, J. W., and Bae, Y. H. (2012). Odyssey of a cancer nanoparticle: from injection site to site of action. *Nano Today* 7, 606–618. doi: 10.1016/j.nantod.2012.10.010

Nichols, J. W., and Bae, Y. H. (2014). EPR: evidence and fallacy. *J. Control. Release* 190, 451–464. doi: 10.1016/j.jconrel.2014.03.057

O'Brien, M. E., Wigler, N., Inbar, M., Rosso, R., Grischke, E., Santoro, A., et al. (2004). Reduced cardiotoxicity and comparable efficacy in a phase III trial of pegylated liposomal doxorubicin HCl (CAELYX/Doxil) versus conventional doxorubicin for first-line treatment of metastatic breast cancer. *Ann. Oncol.* 15, 440–449. doi: 10.1093/annonc/mdh097

Olive, K. P., Jacobetz, M. A., Davidson, C. J., Gopinathan, A., McIntyre, D., Honess, D., et al. (2009). Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* 324, 1457–1461. doi: 10.1126/science.1171362

Olson, P., and Hanahan, D. (2009). Cancer. Breaching the cancer fortress. *Science* 324, 1400–1401. doi: 10.1126/science.1175940

Omary, M. B., Lugea, A., Lowe, A. W., and Pandol, S. J. (2007). The pancreatic stellate cell: a star on the rise in pancreatic diseases. *J. Clin. Invest.* 117, 50–59. doi: 10.1172/JCI30082

Özdemir, B. C., Pentcheva-Hoang, T., Carstens, J. L., Zheng, X., Wu, C. C., Simpson, T. R., et al. (2014). Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. *Cancer Cell* 25, 719–734. doi: 10.1016/j.ccr.2014.04.005

Padera, T. P., Stoll, B. R., Tooredman, J. B., Capen, D., di Tomaso, E., and Jain, R. K. (2004). Pathology: cancer cells compress intratumour vessels. *Nature* 427:695. doi: 10.1038/427695a

Park, J. S., Qiao, L., Su, Z. Z., Hinman, D., Willoughby, K., McKinstry, R., et al. (2001). Ionizing radiation modulates vascular endothelial growth factor (VEGF) expression through multiple mitogen activated protein kinase dependent pathways. *Oncogene* 20, 3266–3280. doi: 10.1038/sj.onc.1204258

Parodi, A., Haddix, S. G., Taghipour, N., Scaria, S., Taraballi, F., Cevenini, A., et al. (2014). Bromelain surface modification increases the diffusion of silica nanoparticles in the tumor extracellular matrix. *ACS Nano* 8, 9874–9883. doi: 10.1021/nn502807n

Peer, D., Karp, J. M., Hong, S., Farokhzad, O. C., Margalit, R., and Langer, R. (2007). Nanocarriers as an emerging platform for cancer therapy. *Nat. Nanotechnol.* 2, 751–760. doi: 10.1038/nnano.2007.387

Pilch, J., Brown, D. M., Komatsu, M., Järvinen, T. A., Yang, M., Peters, D., et al. (2006). Peptides selected for binding to clotted plasma accumulate in tumor stroma and wounds. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2800–2804. doi: 10.1073/pnas.0511219103

Pluen, A., Boucher, Y., Ramanujan, S., McKee, T. D., Gohongi, T., di Tomaso, E., et al. (2001). Role of tumor-host interactions in interstitial diffusion of macromolecules: cranial vs. subcutaneous tumors. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4628–4633. doi: 10.1073/pnas.081626898

Provenzano, P. P., Cuevas, C., Chang, A. E., Goel, V. K., Von Hoff, D. D., and Hingorani, S. R. (2012). Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma. *Cancer Cell* 21, 418–429. doi: 10.1016/j.ccr.2012.01.007

Rapoport, N., Nam, K. H., Gupta, R., Gao, Z., Mohan, P., Payne, A., et al. (2015). Ultrasound-mediated tumor imaging and nanotherapy using drug loaded, block copolymer stabilized perfluorocarbon nanoemulsions. *J. Control. Release* 153, 4–15. doi: 10.1016/j.jconrel.2011.01.022

Rapoport, N., Payne, A., Dillon, C., Shea, J., Scaife, C., and Gupta, R. (2013). Focused ultrasound-mediated drug delivery to pancreatic cancer in a mouse model. *J. Ther. Ultrasound* 1:11. doi: 10.1186/2050-5736-1-11

Ritchie, J. P., Ramani, V. C., Ren, Y., Naggi, A., Torri, G., Casu, B., et al. (2011). SST0001, a chemically modified heparin, inhibits myeloma growth and angiogenesis via disruption of the heparanase/syndecan-1 axis. *Clin. Cancer Res.* 17, 1382–1393. doi: 10.1158/1078-0432.CCR-10-2476

Roode, L. E., Brighton, H., Bo, T., Perry, J. L., Parrott, M. C., Kersey, F., et al. (2016). Subtumoral analysis of PRINT nanoparticle distribution reveals targeting variation based on cellular and particle properties. *Nanomedicine* 12, 1053–1062. doi: 10.1016/j.nano.2015.12.382

Salgaller, M. L. (2003). Technology evaluation: bevacizumab, Genentech/Roche. *Curr. Opin. Mol. Ther.* 5, 657–667.

Schaefer, L., and Schaefer, R. M. (2010). Proteoglycans: from structural compounds to signaling molecules. *Cell Tissue Res.* 339, 237–246. doi: 10.1007/s00441-009-0821-y

Seki, T., Carroll, F., Illingworth, S., Green, N., Cawood, R., Bachtarzi, H., et al. (2011). Tumour necrosis factor-alpha increases extravasation of virus particles into tumour tissue by activating the Rho A/Rho kinase pathway. *J. Control. Release* 156, 381–389. doi: 10.1016/j.jconrel.2011.08.022

Seki, T., Fang, J., and Maeda, H. (2009). Enhanced delivery of macromolecular antitumor drugs to tumors by nitroglycerin application. *Cancer Sci.* 100, 2426–2430. doi: 10.1111/j.1349-7006.2009.01323.x

Sen, A., Capitano, M. L., Spernyak, J. A., Schueckler, J. T., Thomas, S., Singh, A. K., et al. (2011). Mild elevation of body temperature reduces tumor interstitial fluid pressure and hypoxia and enhances efficacy of radiotherapy in murine tumor models. *Cancer Res.* 71, 3872–3880. doi: 10.1158/0008-5472.CAN-10-4482

Sevick, E. M., and Jain, R. K. (1989a). Geometric resistance to blood flow in solid tumors perfused *ex vivo*: effects of tumor size and perfusion pressure. *Cancer Res.* 49, 3506–3512.

Sevick, E. M., and Jain, R. K. (1989b). Viscous resistance to blood flow in solid tumors: effect of hematocrit on intratumor blood viscosity. *Cancer Res.* 49, 3513–3519.

Sherman, M. H., Yu, R. T., Engle, D. D., Ding, N., Atkins, A. R., Tiriac, H., et al. (2014). Vitamin D receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy. *Cell* 159, 80–93. doi: 10.1016/j.cell.2014.08.007

Shiga, K., Hara, M., Nagasaki, T., Sato, T., Takahashi, H., and Takeyama, H. (2015). Cancer-associated fibroblasts: their characteristics and their roles in tumor growth. *Cancers (Basel).* 7, 2443–2458. doi: 10.3390/cancers7040902

Smith, N. R., Baker, D., Farren, M., Pommier, A., Swann, R., Wang, X., et al. (2013). Tumor stromal architecture can define the intrinsic tumor response to VEGF-targeted therapy. *Clin. Cancer Res.* 19, 6943–6956. doi: 10.1158/1078-0432.CCR-13-1637

Sonveaux, P., Dessy, C., Martinive, P., Havaux, X., Jordan, B. F., Gallez, B., et al. (2004). Endothelin-1 is a critical mediator of myogenic tone in tumor arterioles: implications for cancer treatment. *Cancer Res.* 64, 3209–3214. doi: 10.1158/0008-5472.CAN-03-1291

Stapleton, S., Jaffray, D., and Milosevic, M. (2016). Radiation effects on the tumor microenvironment: implications for nanomedicine delivery. *Adv. Drug Deliv. Rev.* 109, 119–130. doi: 10.1016/j.addr.2016.05.021

Stylianopoulos, T., and Jain, R. K. (2013). Combining two strategies to improve perfusion and drug delivery in solid tumors. *Proc. Natl. Acad. Sci. U.S.A.* 110, 18632–18637. doi: 10.1073/pnas.1318415110

Stylianopoulos, T., Martin, J. D., Chauhan, V. P., Jain, S. R., Diop-Frimpong, B., Bardeesy, N., et al. (2012). Causes, consequences, and remedies for growth-induced solid stress in murine and human tumors. *Proc. Natl. Acad. Sci. U.S.A.* 109, 15101–15108. doi: 10.1073/pnas.1213353109

Stylianopoulos, T., Yeckel, A., Derby, J. J., Luo, X. J., Shephard, M. S., Sander, E. A., et al. (2008). Permeability calculations in three-dimensional isotropic and oriented fiber networks. *Phys. Fluids (1994).* 20, 123601. doi: 10.1063/1.3021477

Sun, C., Jain, R. K., and Munn, L. L. (2007). Non-uniform plasma leakage affects local hematocrit and blood flow: implications for inflammation and tumor perfusion. *Ann. Biomed. Eng.* 35, 2121–2129. doi: 10.1007/s10439-007-9377-8

Tanaka, S., Akaike, T., Wu, J., Fang, J., Sawa, T., Ogawa, M., et al. (2003). Modulation of tumor-selective vascular blood flow and extravasation by the stable prostaglandin 12 analogue beraprost sodium. *J. Drug Target.* 11, 45–52. doi: 10.1080/1061186031000086072

Tang, L., Yang, X., Yin, Q., Cai, K., Wang, H., Chaudhury, I., et al. (2014). Investigating the optimal size of anticancer nanomedicine. *Proc. Natl. Acad. Sci. U.S.A.* 111, 15344–15349. doi: 10.1073/pnas.1411499111

Thakkar, D., Gupta, R., Monson, K., and Rapoport, N. (2013). Effect of ultrasound on the permeability of vascular wall to nano-emulsion droplets. *Ultrasound Med. Biol.* 39, 1804–1811. doi: 10.1016/j.ultrasmedbio.2013.04.008

Tong, R. T., Boucher, Y., Kozin, S. V., Winkler, F., Hicklin, D. J., and Jain, R. K. (2004). Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. *Cancer Res.* 64, 3731–3736. doi: 10.1158/0008-5472.CAN-04-0074

Tong, R., Chiang, H. H., and Kohane, D. S. (2013). Photoswitchable nanoparticles for *in vivo* cancer chemotherapy. *Proc. Natl. Acad. Sci. U.S.A.* 110, 19048–19053. doi: 10.1073/pnas.1315336110

Turley, R. S., Fontanella, A. N., Padussis, J. C., Toshimitsu, H., Tokuhisa, Y., Cho, E. H., et al. (2012). Bevacizumab-induced alterations in vascular permeability and drug delivery: a novel approach to augment regional chemotherapy for in-transit melanoma. *Clin. Cancer Res.* 18, 3328–3339. doi: 10.1158/1078-0432.CCR-11-3000

von Maltzahn, G., Park, J. H., Lin, K. Y., Singh, N., Schwöppe, C., Mesters, R., et al. (2011). Nanoparticles that communicate *in vivo* to amplify tumour targeting. *Nat. Mater.* 10, 545–552. doi: 10.1038/nmat3049

Wang, X., Li, H., Liu, X., Tian, Y., Guo, H., Jiang, T., et al. (2017). Enhanced photothermal therapy of biomimetic polypyrrole nanoparticles through improving blood flow perfusion. *Biomaterials* 143, 130–141. doi: 10.1016/j.biomaterials.2017.08.004

Winslow, T. B., Eranki, A., Ullas, S., Singh, A. K., Repasky, E. A., and Sen, A. (2015). A pilot study of the effects of mild systemic heating on human head and neck tumour xenografts: analysis of tumour perfusion, interstitial fluid pressure, hypoxia and efficacy of radiation therapy. *Int. J. Hyperthermia* 31, 693–701. doi: 10.3109/02656736.2015.1037800

Wong, C., Stylianopoulos, T., Cui, J., Martin, J., Chauhan, V. P., Jiang, W., et al. (2011). Multistage nanoparticle delivery system for deep penetration into tumor tissue. *Proc. Natl. Acad. Sci. U.S.A.* 108, 2426–2431. doi: 10.1073/pnas.1018382108

Wong, K. M., Horton, K. J., Coveler, A. L., Hingorani, S. R., and Harris, W. P. (2017). Targeting the tumor stroma: the biology and clinical development of pegylated recombinant human hyaluronidase (PEGPH20). *Curr. Oncol. Rep.* 19:47. doi: 10.1007/s11912-017-0608-3

Yoon, Y. I., Kwon, Y. S., Cho, H. S., Heo, S. H., Park, K. S., Park, S. G., et al. (2014). Ultrasound-mediated gene and drug delivery using a microbubble-liposome particle system. *Theranostics* 4, 1133–1144. doi: 10.7150/thno.9945

Yuan, F., Leunig, M., Huang, S. K., Berk, D. A., Papahadjopoulos, D., and Jain, R. K. (1994a). Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res.* 54, 3352–3356.

Yuan, F., Salehi, H. A., Boucher, Y., Vasthare, U. S., Tuma, R. F., and Jain, R. K. (1994b). Vascular permeability and microcirculation of gliomas and mammary

carcinomas transplanted in rat and mouse cranial windows. *Cancer Res.* 54, 4564–4568.

Zhang, B., Jiang, T., Ling, L., Cao, Z., Zhao, J., Tuo, Y., et al. (2016a). Enhanced Antitumor Activity of EGFP-EGF1-Conjugated Nanoparticles by a Multitargeting Strategy. *ACS Appl. Mater. Interfaces* 8, 8918–8927. doi: 10.1021/acsami.6b00036

Zhang, B., Jiang, T., She, X., Shen, S., Wang, S., Deng, J., et al. (2016b). Fibrin degradation by rtPA enhances the delivery of nanotherapeutics to A549 tumors in nude mice. *Biomaterials* 96, 63–71. doi: 10.1016/j.biomaterials.2016.04.015

Zhang, B., Jiang, T., Shen, S., She, X., Tuo, Y., Hu, Y., et al. (2016c). Cyclopamine disrupts tumor extracellular matrix and improves the distribution and efficacy of nanotherapeutics in pancreatic cancer. *Biomaterials* 103, 12–21. doi: 10.1016/j.biomaterials.2016.06.048

Zhang, B., Jiang, T., Tuo, Y., Jin, K., Luo, Z., Shi, W., et al. (2017a). Captopril improves tumor nanomedicine delivery by increasing tumor blood perfusion and enlarging endothelial gaps in tumor blood vessels. *Cancer Lett.* 410, 12–19. doi: 10.1016/j.canlet.2017.09.007

Zhang, B., Jin, K., Jiang, T., Wang, L., Shen, S., Luo, Z., et al. (2017b). Celecoxib normalizes the tumor microenvironment and enhances small nanotherapeutics delivery to A549 tumors in nude mice. *Sci. Rep.* 7:10071. doi: 10.1038/s41598-017-09520-7

Zhang, B., Shen, S., Liao, Z., Shi, W., Wang, Y., Zhao, J., et al. (2014a). Targeting fibronectins of glioma extracellular matrix by CLT1 peptide-conjugated nanoparticles. *Biomaterials* 35, 4088–4098. doi: 10.1016/j.biomaterials.2014.01.046

Zhang, B., Shi, W., Jiang, T., Wang, L., Mei, H., Lu, H., et al. (2016d). Optimization of the tumor microenvironment and nanomedicine properties simultaneously to improve tumor therapy. *Oncotarget* 7, 62607–62618. doi: 10.18632/oncotarget.11546

Zhang, B., Wang, H., Liao, Z., Wang, Y., Hu, Y., Yang, J., et al. (2014b). EGFP-EGF1-conjugated nanoparticles for targeting both neovascular and glioma cells in therapy of brain glioma. *Biomaterials* 35, 4133–4145. doi: 10.1016/j.biomaterials.2014.01.071

Zhang, B., Zhang, Y., Liao, Z., Jiang, T., Zhao, J., Tuo, Y., et al. (2015). UPA-sensitive ACPP-conjugated nanoparticles for multi-targeting therapy of brain glioma. *Biomaterials* 36, 98–109. doi: 10.1016/j.biomaterials.2014.09.008

Zhang, J., Miao, L., Guo, S., Zhang, Y., Zhang, L., Satterlee, A., et al. (2014). Synergistic anti-tumor effects of combined gemcitabine and cisplatin nanoparticles in a stroma-rich bladder carcinoma model. J. *Control Release* 182, 90–96. doi: 10.1016/j.jconrel.2014.03.016

Zhou, H., Fan, Z., Deng, J., Lemons, P. K., Arhontoulis, D. C., Bowne, W. B., et al. (2016). Hyaluronidase embedded in nanocarrier PEG shell for enhanced tumor penetration and highly efficient antitumor efficacy. *Nano Lett.* 16, 3268–3277. doi: 10.1021/acs.nanolett.6b00820

Zhu, L., Kate, P., and Torchilin, V. P. (2012). Matrix metalloprotease 2-responsive multifunctional liposomal nanocarrier for enhanced tumor targeting. *ACS Nano* 6, 3491–3498. doi: 10.1021/nn300524f

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REVIEW
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Targeting Accessories to the Crime: Nanoparticle Nucleic Acid Delivery to the Tumor Microenvironment

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Nucleic acid delivery for cancer holds extraordinary promise. Increasing expression of tumor suppressor genes or inhibition of oncogenes in cancer cells has important therapeutic potential. However, several barriers impair progress in cancer gene delivery. These include effective delivery to cancer cells and relevant intracellular compartments. Although viral gene delivery can be effective, it has the disadvantages of being immunostimulatory, potentially mutagenic and lacking temporal control. Various nanoparticle (NP) platforms have been developed to overcome nucleic acid delivery hurdles, but several challenges still exist. One such challenge has been the accumulation of NPs in non-cancer cells within the tumor microenvironment (TME) as well as the circulation. While uptake by these cancer-associated cells is considered to be an off-target effect in some contexts, several strategies have now emerged to utilize NP-mediated gene delivery to intentionally alter the TME. For example, the similarity of NPs in shape and size to pathogens promotes uptake by antigen presenting cells, which can be used to increase immune stimulation and promote tumor killing by T-lymphocytes. In the era of immunotherapy, boosting the ability of the immune system to eliminate cancer cells has proven to be an exciting new area in cancer nanotechnology. Given the importance of cancer-associated cells in tumor growth and metastasis, targeting these cells in the TME opens up new therapeutic applications for NPs. This review will cover evidence for non-cancer cell accumulation of NPs in animal models and patients, summarize characteristics that promote NP delivery to different cell types, and describe several therapeutic strategies for gene modification within the TME.

Keywords: gene delivery, cancer, nanomaterials, immuno-oncology, cell targeting

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INTRODUCTION

Over the last several decades, impressive advancements in nucleic acid delivery have brought these technologies to the clinic. Three gene therapies have been approved by the U.S. Food and Drug Administration (FDA), all of which rely on viral delivery systems. Non-viral systems are less immunogenic than viral systems, which may be required in cases where repeat administration is necessary, and they are non-mutagenic. While stable viral integration of genes may be a beneficial

treatment for genetic disorders, a more transient regulation of gene expression may be preferred in other contexts. Non-viral nucleic acid delivery has not yet reached FDA approval, but several nanoparticles (NP)-based therapeutics are currently in clinical trials. For a thorough review of non-viral nucleic acid therapies in clinical development, we suggest (Yin et al., 2014). As these delivery platforms reach regulatory approval in the United States and elsewhere, they will pave the way for nucleic acid therapeutics in cancer and other diseases.

In parallel with advancements in nucleic acid delivery, the development of immunotherapies has revolutionized cancer treatment. Although historically cancer therapies have focused on directly killing cancer cells through chemotherapy and radiation, the success of immune checkpoint inhibitors and chimeric antigen receptor (CAR) T-cells has demonstrated that turning the immune tumor microenvironment (TME) against cancers can have strong therapeutic effects (Hoos, 2016). However, these immune-oncology drugs are only effective for subsets of patients, suggesting that additional factors are at play. An immune suppressive TME is one critical factor that can hamper T-cell invasion and anti-tumor effects. Taken together, harnessing NP-based nucleic acid delivery to the TME could transform a pro-tumoral and immuno-suppressive TME into a toxic environment for cancer cells. Here we review pre-clinical studies that demonstrate the feasibility of nucleic acid delivery to the TME for cancer therapy.

NANOPARTICLES FOR NUCLEIC ACID DELIVERY

Naked nucleic acids display unfavorable biodistribution and pharmacokinetics: once injected into the blood stream, RNA and DNA can be quickly degraded by nucleases, phagocytosed by immune cells in the blood, or excreted through the kidneys. Therefore, to be effective, nucleic acids require delivery vehicles (Yin et al., 2014). All nucleic acids share a similar chemical structure: repeated nucleotides each composed of a five-carbon sugar linked to a nitrogenous base and connected by a phosphate backbone. While nucleic acids vary in size and contain either ribose or deoxyribose (in RNA and DNA, respectively), they are all negatively charged and hydrophilic. These properties allow them to be efficiently encapsulated into NPs. NPs are a diverse group of biomaterials that form structures in the nanometer scale. These include particles made of gold, silica, polymers, lipids, and others. While there are exceptions, lipids and polymers are the most common materials used for delivery of nucleic acids. For example, most commercially available transfection reagents use cationic lipids for effective intracellular delivery of DNA and RNAs such as mRNA, microRNA (miRNA) and short interfering RNAs (siRNAs) *in vitro*. In large part, *in vivo* nucleic acid delivery relies on similar principles but faces additional barriers such as stability in the circulation and delivery to target cells.

Lipids

Lipid systems for *in vitro* gene delivery were first developed in the 1980s and were primarily composed of amphiphilic cationic lipids (Felgner et al., 1987). These molecules contain a polar head group, linker, and fatty acid chains that self-assemble into micellar, lamellar, or hexagonal structures in water: examples include N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP). Incorporation of cholesterol and neutral lipids such as 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) can also increase stability and transfection efficiency. Permanently charged lipids result in toxicity, therefore ionizable systems have been developed. Ionizable lipids are positively charged in mildly acidic conditions where they can complex with nucleic acids; however, they remain uncharged at neutral pH which avoids systemic toxicity (Rietwyk and Peer, 2017).

Polymers

Polymers can also be used to encapsulate nucleic acids for *in vivo* delivery. Generally, polymers can be divided into two groups: natural or synthetic. Biologically occurring molecules such as peptides, oligosaccharides, and even nucleic acids themselves are natural polymers. Synthetic polymers are chemically produced, such as poly(lactic-co-glycolic acid) (PLGA). Polymers can occur as a single repeating unit (homopolymers) or multiple unit types (copolymers). Additionally, polymers with discrete segments consisting of different repeating units, called block-copolymers can be made with a variety of useful properties. The cationic polymers poly-L-lysine (PLL) and polyethylenimine (PEI) were the earliest polymers used for condensing DNA. PEI has superior transfection efficiency and has been developed for *in vivo* and clinical delivery of nucleic acids (Yin et al., 2014). Combining PLGA, which is safe, biodegradable, and forms stable NPs, with PEI into mixed polymer NPs allows for effective gene delivery *in vivo* (Bivas-Benita et al., 2004). The neutral polymer polyethylene glycol (PEG) has also been used to reduce electrostatic interactions of both polymer and lipid-based NPs *in vivo* (Storm et al., 1995). Another nucleic acid delivery agent gaining popularity is chitosan, a natural co-polymer that can act as a biodegradable gene delivery agent (Wang et al., 2011). There are a wide variety of materials used in nanoformulations for nucleic acid delivery. The combination of these diverse materials and formulation techniques results in NPs with an array of sizes, charges, and surface properties. The properties of NPs used for nucleic acid delivery to the TME in preclinical studies are summarized in **Table 1**.

NP Delivery to Tumors

In the case of solid tumors, delivery to cancer cells is a formidable hurdle (Pecot et al., 2011), but evidence of tolerability and intracellular delivery has been demonstrated in phase I clinical trials for both lipid and polymeric NPs (Zuckerman and Davis, 2015). While leaky and inefficient vasculature can allow accumulation of NPs in the tumor (Prabhakar et al., 2013), solid tumors also have stroma that contains a vast milieu of non-cancerous constituents that include fibroblasts, tumor-associated macrophages (TAMs), endothelial cells and extracellular matrix components (ECM) that additionally impair access to the tumor parenchyma. In the case of NPs loaded with small molecule drugs,

TABLE 1 | Characteristics of nanoparticles used to target the tumor microenvironment in pre-clinical models.

Target cell type	Type of nucleic acid	Target gene(s)	Formulation material	Targeting moiety	Size (nm)	Surface charge (mV)	Reference
Macrophage	siRNA	*PPIB*	Amphipathic triblock copolymers (polymeric micelle)	Mannose	30	+20	Shann et al., 2013
	Cy5-labeled dsDNA	Na	Amphipathic triblock copolymers (polymeric micelle)	Mannose	nr	+20	Ortega et al., 2015
	siRNA	*IκBα*	Amphipathic triblock copolymers (polymeric micelle)	Mannose	nr	nr	Ortega et al., 2016
	siRNA and CpG oligonucleotide	*IL-10, IL-10RA*	PEGylated polymeric NP (glucan)	Galactose	270	nr	Huang et al., 2012
	miRNA	miR-155	PEGylated polymeric NP (polypeptide)	Galactose	100	+5	Liu L. et al., 2017
	siRNA	*CSF-1R*	PEGylated cationic phospholipid monolayer	ApoA 1-mimetic α-helical peptide linked to M2 macrophage binding protein	20	nr	Qian et al., 2017
	siRNA	*MIF*	Polymeric NP (glucan)	Glucan	nr	nr	Zhang et al., 2015b
	siRNA	*MIF*	Polymeric NP (glucan)	Glucan	80–120	nr	Zhang et al., 2015a
Monocyte	siRNA	*CCR2*	PEGylated cationic liposome	na	70–80	nr	Leuschner et al., 2011
Dendritic cell	siRNA	*PD-L1*	Polymeric NP (PEI)	na	nr	nr	Cubillos-Ruiz et al., 2009
	siRNA	*SOCS1*	Polymeric NP (PLGA-PLL)	na	150	−29	Heo et al., 2014
	siRNA	*STAT3*	Polymeric NP (PLGA-PLL)	na	100–200	−24	Heo and Lim, 2014
	siRNA or CpG oligonucleotide	*IL-10*	Polymeric NP (PLGA-PLL)	na	100–200	−20	Heo et al., 2015
	siRNA	*XBP1 or IRE1*	Polymeric NP (PEI)	na	nr	nr	Cubillos-Ruiz et al., 2015
	miRNA	miR-155	Polymeric NP (PEI)	na	nr	nr	Cubillos-Ruiz et al., 2012
Cancer-associated fibroblast	siRNA	*Wnt16*	PEGylated cationic liposome	Amino-ethyl anisamide	50	+25	Miao et al., 2015
	pDNA	soluble TRAIL	PEGylated cationic liposome	Amino-ethyl anisamide	70	+25	Miao et al., 2017b
	pDNA	PD-L1 and CXCL12 traps	PEGylated cationic liposome	Amino-ethyl anisamide	70	nr	Miao et al., 2017a
T cells	mRNA	megaTAL nuclease, *TREX2, Foxo1*$_{3A}$	Polymeric NP (PBAE)	Anti-CD3 and anti-CD8 antibodies	110	+1	Moffett et al., 2017
Blood vessel endothelial cells	siRNA	*EZH2*	Polymeric NP (Chitosan-TPP)	na	100–200	+35	Lu et al., 2010
	Anti-miR	miR-132	Cationic liposome	αVβ3 ligand	100–200	nr	Anand et al., 2010
	miRNA	miR-200a and b	Polymeric NP (Chitosan-TPP)	RGD	100–200	nr	Pecot et al., 2013
	siRNA	*POSTN, FAK, PLXDC1*	Polymeric NP (Chitosan-TPP)	RGD	100–200	+40	Hee-Dong et al., 2010
	pDNA	*ATPμ-Raf*	Cationic liposome	αVβ3 ligand	100–200	+35	Hood et al., 2002
	siRNA	*CD31, Tie2*	PEGylated cationic liposome	na	100–200	nr	Santel et al., 2006
	siRNA	*VEGFR-1 and Dll4*	PEGylated lipid–polymer hybrid NP	na	100–200	0	Dahlman et al., 2014

PEG, polyethylene glycol; PLL, poly-L-lysine; PEI, Polyethylenimine; TPP, tripolyphosphate; PLGA, poly(lactic-co-glycolic acid); PBAE, poly(β-aminoester); na, not applicable; nr, not reported.

delivery to TAMs in the TME may be beneficial for local and sustained release of drug (Miller et al., 2015). This is not the case for nucleic acid delivery, where not only is delivery to appropriate cells required, but the payload must also reach key intracellular compartments. While strategies to allow better penetrance of NPs through the tumor stroma are being explored, caution is warranted. Disruption of tumor stroma may remove important elements of nutrition and growth factors, but it can also promote resistance (Miao et al., 2015). One alternative strategy is to target tumor-associated cells within the TME for cancer therapy. Commonly found in the tumor periphery, these cells are the first to encounter NPs as they leave the circulation. Many cell types within the TME also express unique cell surface markers, which can be utilized for targeted delivery. Given the influence of TME cells on all of the hallmarks of cancer, this is an enticing direction to pursue (Hanahan and Coussens, 2012). TME cell types, their role in cancer biology, and surface markers commonly used to target them are summarized in **Figure 1**.

TYPES OF NUCLEIC ACIDS FOR NP DELIVERY

DNA

Delivery of exogenous DNA offers a great therapeutic opportunity for cancer. One approach is overexpression of genes that can suppress or kill tumor cells. These genes can be human in origin, viral proteins, bacterial toxins, or proteins designed for desired functions. One such example is NP delivery of the gene encoding anti-tumoral viral protein E1A, which was successfully delivered and expressed in humans (Chang et al., 1997; Yoo et al., 2001; Ueno et al., 2002). Many barriers to gene delivery are universal to all oligonucleotide species: stability in circulation, cell uptake, and endosomal escape. DNA must additionally be delivered to the nuclear compartment to permit access to transcriptional machinery. It was shown over 30 years ago that DNA microinjected into the nucleus, but not the cytosol, produces a gene product (Capecchi, 1980). Improved delivery systems and alterations in DNA sequences can enhance nuclear delivery. For example, import into the nucleus can be aided by inclusion of a nuclear localization signal (NLS) in the plasmid DNA (pDNA) sequence (Brandén et al., 1999; Zanta et al., 1999). Also, strong viral or eukaryotic promoter sequences can be added to activate transcription (Capecchi, 1980). Plasmids can be further tailored by using tissue-specific promoters to reduce potential off-target effects (Gorski et al., 1986).

Important safety concerns for DNA delivery are immune stimulation and risk of insertional mutagenesis. The risk of insertional mutagenesis is much higher for viral delivery systems, but cannot be excluded for episomal pDNA (Glover et al., 2005; Baum et al., 2006). Like other oligonucleotides, DNA can stimulate immune responses that should be considered when designing therapeutics. Immune responses to DNA are caused by unmethylated CpG motifs which stimulate B-cell proliferation and cytokine release through TLR9 (Krieg et al., 1995; Klinman et al., 1996; Hemmi et al., 2000). The robust immunostimmulatory effect of CpG DNA makes it a potentially useful vaccine adjuvant (Gurunathan et al., 2000). Alternatively, the immune effects of CpG can also be removed by altering the DNA sequence to replace CpG with CpG-S motifs (Krieg et al., 1998). Thorough reviews of DNA delivery technology platforms have been published elsewhere (Nishikawa and Huang, 2001; Yin et al., 2014).

Delivery of DNA encoding a gene of interest is a powerful tool for gene therapy and important advances toward this goal have been made. While no therapies have been approved by the FDA thus far, several non-viral DNA strategies are being evaluated clinically, including in clinical trials for cancer therapy. In a phase 1 clinical trial, the tumor suppressor gene *TUSC2* was delivered to lung cancer patients using DOTAP-cholesterol liposomes, resulting in transgene expression and activation of apoptotic pathways (Lu et al., 2012). This therapy is now in phase 1/2 trials. Other gene delivery trails include IL-12 gene delivery in PEG–PEI–cholesterol NPs to enhance immune response (Kendrick et al., 2008; Anwer et al., 2010) and co-delivery of two tumor suppressor genes somatostatin receptor subtype 2 (SSTR2) and deoxycytidine kinase::uridylmonophosphate kinase (DCK::UMK) complexed to PEI (Buscail et al., 2015). Delivery of the cytotoxic diphtheria toxin A gene as a "suicide" gene under cancer specific promoters in PEI NPs is also being evaluated in ovarian, pancreatic, and bladder cancer and has demonstrated good safety profiles and anti-tumor efficacy (Sidi et al., 2008; Smaldone and Davies, 2010; Hanna et al., 2012; Gofrit et al., 2014). If these non-viral DNA therapeutics show substantial efficacy in clinical trials, they will pave the way for non-viral DNA in cancer therapy and other diseases. After 3 decades of technology development, therapeutic DNA delivery in humans is becoming a reality.

mRNA

The goal of mRNA delivery is the same as DNA delivery, to deliver a therapeutic gene that will be translated into protein within target cells. In contrast to DNA, mRNA needs to reach the cytosol and be recognized by ribosomes. Transfection efficiency with mRNA is higher than for DNA, especially in non-dividing cells (Yamamoto et al., 2009). Also, mRNA delivery does not pose the risk of insertional mutagenesis. Although stability of naked mRNA is poor, chemical modifications and protection from serum endonucleases in NP delivery vehicles can increase mRNA stability. As with DNA, exogenous mRNA can also stimulate an immune response through TLR3, TLR7, TLR8, and retinoic acid receptor responder protein 3 (RARRES3 or RIG-I) (Yin et al., 2014). Chemical modifications can reduce recognition of mRNA by the immune system (Karikó et al., 2005).

The structure of mRNA is critical for recognition by the eukaryotic translation machinery. At the core of the mRNA structure is an open reading frame (ORF) that is translated into protein. Flanking the ORF are two untranslated regions (UTRs) at the 3′ and 5′ ends that allow for regulation of translation. Finally, a 5′ methyl cap and a 3′ poly adenosine tail book-end the mRNA and are necessary for efficient translation (Gallie, 1991). Increasing the poly(A) tail length also improves stability (Holtkamp et al., 2006). Commercial kits are available to synthesize mRNA with all necessary structural components from

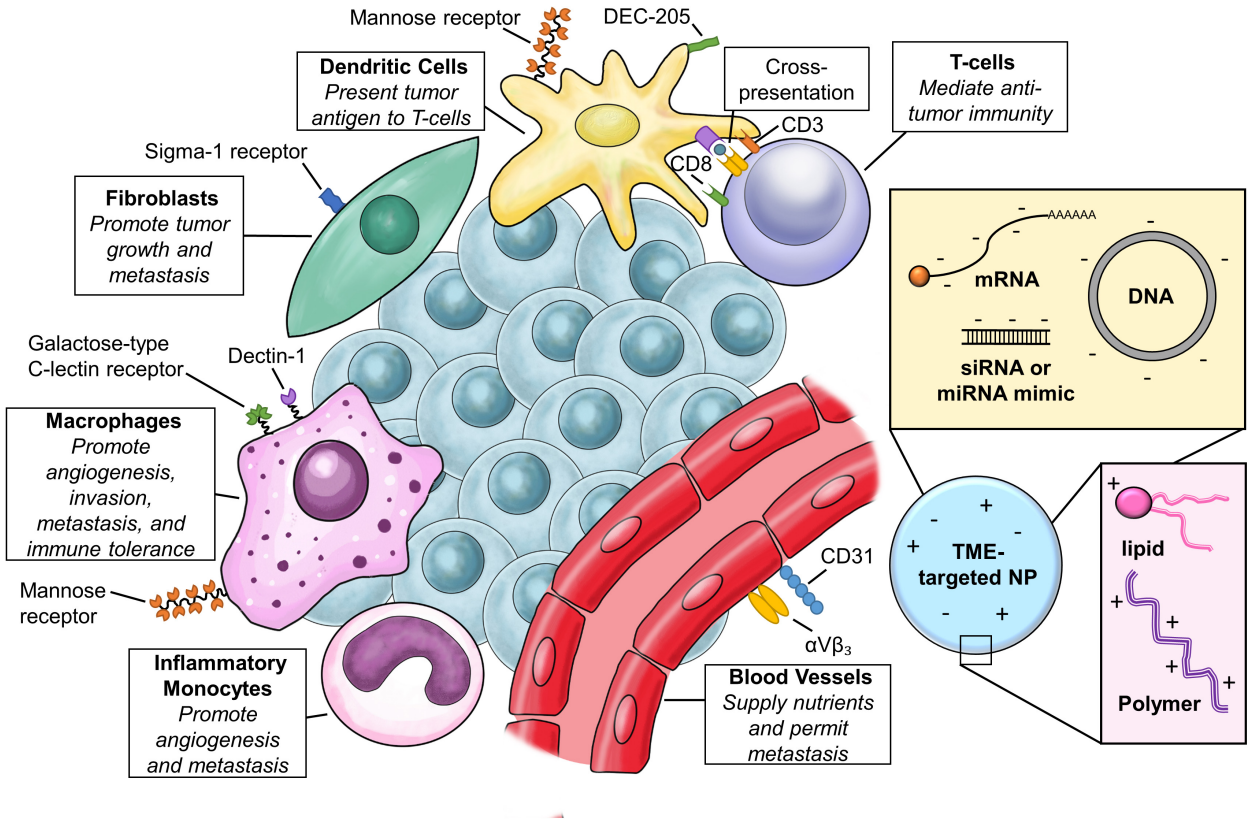

FIGURE 1 | Targeting the tumor microenvironment (TME). Relevant cell types in the TME, common cell surface receptors for targeted delivery, and basic composition of nanoparticles (NPs) for TME targeting.

plasmid DNA, though optimization for target cell types can also help improve translation efficiency (Yamamoto et al., 2009).

There are over a dozen clinical trials using mRNA for vaccines, adjuvants, or to express antigens either in dendritic cells *ex vivo* or by direct injection. However, gene replacement therapy is still in pre-clinical development (Kaczmarek et al., 2017).

MicroRNAs (miRNAs)

Discovered in 1993, miRNAs are a class of non-coding, regulatory RNAs that have critical roles in nearly all biological processes, including cancer. miRNAs can serve as both oncogenes and tumor suppressors (Farazi et al., 2013). Primary miRNA transcripts have characteristic hairpin structures that are recognized and processed by RNase III enzyme Drosha, which produces a stem loop precursor miRNA (pre-miRNA) of ∼70 nucleotides (Lee et al., 2003). Final cleavage by Dicer results in a mature dsRNA (Hutvágner et al., 2001). The mature ∼22 nucleotide miRNA associates with the RNA-induced silencing complex (RISC), and one or both strands of the duplex guide RISC to complementary sequences within target mRNA. Strand selection by RISC is likely based on stability. The two strands are named 5p and 3p corresponding to the 5′ and 3′ ends of the miRNA precursor hairpin, respectively. Target sequences complimentary to the 2–7 nucleotide "seed" region of the miRNA sequence are frequently found in the 3′ UTR of mRNAs, but can also be within coding or intronic regions. Binding of miRNA to target mRNA results in degradation or destabilization of the mRNA and can also cause translational repression (Ha and Kim, 2014). Generally speaking, each miRNA can target hundreds of unique mRNAs, and thus can regulate transcriptome-wide changes. In this way, miRNAs are critical regulators of cell identity and state (Kosik, 2010). Additionally, miRNAs are essential for immune cell development and immune activation (Xiao and Rajewsky, 2009) as well as cross-talk between cancer cells and the TME (Chou et al., 2013).

To replace a downregulated miRNA, synthetic double-stranded RNAs (dsRNAs) carrying the same sequence as the endogenous mature or precursor miRNA can be used. These miRNA "mimics" are smaller and more stable than mRNA, allowing ease of encapsulation in several types of NPs. Chemical modifications to miRNAs can be made in the same way as mRNAs or siRNAs to increase stability and reduce inflammatory response. Nucleic acid based inhibitors of miRNAs include locked-nucleic acids (LNAs), antagomirs, anti-miRs, and miR-sponges have been characterized in more detail elsewhere (Ling et al., 2013). Therapeutic strategies modulating miRNA function are already in clinical trials, and a comprehensive review of miRNA therapeutics is provided elsewhere (Rupaimoole and Slack, 2017). Inhibiting miR-122, a critical player for hepatitis infection is being investigated in multiple clinical trials, and

inhibition of miR103/107 is in clinical trials for alcoholic fatty liver disease. Both drugs utilize unencapsulated anti-miRs. While these therapeutics target the liver, a relatively accessible organ target for nucleic acid delivery, trials are also in progress for cancer therapy. Delivery of miR-16 with EGFR-targeted EnGeneIC Delivery Vehicle nanocells completed phase 1 clinical trials in mesothelioma with an acceptable safety profile and signs of efficacy (van Zandwijk et al., 2017). In contrast, phase 1 trials for miR-34 mimics for multiple solid tumors were recently terminated due to severe immune-related and marrow suppressive adverse events. A 110 nm liposomal carrier composed of ionizable lipids was used in these trials. The precise source of the inflammatory reactions—be it due to the carrier, miRNA mimic, or synergy between the two—is not known (Beg et al., 2017; Rupaimoole and Slack, 2017). It is clear that going forward, extensive pre-clinical evaluation of immune stimulation by miRNA-directed therapies must to be an important consideration.

siRNA

siRNAs are ∼21 nt dsRNAs that interact in the cytoplasm with the RISC complex to degrade target mRNAs. In structure, siRNAs are identical to miRNAs and can be modified and encapsulated in the same way. Other than the fact that miRNA mimics are based on endogenous genes while siRNAs are synthetically designed, the primary difference between miRNAs and siRNAs is the sequence specificity. Instead of a miRNA seed region of 6–8 nt with complementarity to the 3-UTR of target mRNAs, by design siRNAs usually have 100% complementarity to target mRNAs. As such, siRNAs have potent activity on a single target. In contrast, miRNAs have the potential to target hundreds of mRNAs, but generally this inhibition is to a lesser extent. The targets of siRNAs are degraded by the endonuclease activity of Argonaut 2 associated with the RISC, while miRNAs usually cause deadenylation or translational repression of target mRNAs by association with other Argonaut complexes devoid of nuclease activity. Therefore, the pharmacodynamics of miRNA mimics and siRNAs have important differences. However, the pharmacokinetics and biodistribution of these two oligonucleotides is the same, since their chemical structure is identical. Another consideration is stimulation of immune responses by exogenous RNA. For instance, siRNAs within liposomal or polymeric NPs can induce an inflammatory cytokine response that is greater than either component alone. The response is mediated by TLRs and is sequence dependent, with GU-rich sequences inducing the most potent cytokine response (Judge et al., 2005). Modifications of RNA, such as incorporation of 2′-O-methyl nucleosides, can reduce inflammatory response without decreasing gene silencing (Judge et al., 2006). Inflammatory cytokine responses have been observed in siRNA and miRNA clinical trials, and therefore evaluating immunostimulation should be a concern when translating these therapeutics to the clinic. Currently, dozens of clinical trials of siRNA-based therapies have been completed or are ongoing, for in depth review see Wittrup and Lieberman (2015) and Zuckerman and Davis (2015).

Other Non-coding RNAs

Several classes of non-coding RNAs have been identified including piwi-interacting RNAs, endogenous siRNAs, long-non-coding RNAs, and circular RNAs. The function of these species and their role in disease biology are being actively investigated (Esteller, 2011). As the role of non-coding RNAs in cancer biology continues to unfold, therapeutic approaches to modulate them will be of increasing interest (Gutschner and Diederichs, 2012). Therapeutic delivery of non-coding RNAs will face similar challenges to other types of RNA, such as stability and uptake into appropriate cells and intracellular compartments.

Genome Editing

The targeted manipulation of genomic DNA in living cells is possible through the use of engineered nucleases, such as mega nucleases, zinc finger nucleases (ZFN), transcription activator-like effector-based nucleases (TALEN), and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system (Cox et al., 2015). The enthusiasm surrounding genome editing technology is reminiscent of that generated by the discovery of RNAi (Fire et al., 1998). However, similar hurdles impede therapeutic translation. To enact genome editing *in vivo* nucleases must be present within target cells. While delivery of protein may be possible, most approaches utilize delivery of mRNA or DNA encoding nucleases. Using viral vectors, such as AAV, to deliver nucleases raises additional concerns. Integration of nuclease DNA leads to constitutive expression of nucleases capable of genome editing and increases the chances of off-target mutations. For this reason, transient expression – as is achieved with NP systems-may be preferable.

CRISPR-Cas9 is a highly specific gene editing tool that is rapidly becoming a standard lab technique. In its simplest embodiment, it requires the presence of a Cas9 protein and single guide RNA (sgRNA) to be present in the same cell (Liu C. et al., 2017). The first use of CRISPR-Cas9 in clinical trials has already taken place. In this trial the T-cell exhaustion receptor PD-1 was genetically removed *ex vivo* by CRISPR-Cas9 technology and cells were then delivered to the patient (Cyranoski, 2016). Similarly, CRISPR-Cas9 is being used to remove the CCR5 receptor from hematopoietic stem cells as a therapeutic strategy for HIV. Since HIV enters cells through CCR5, removing this receptor is expected to reduce the ability of HIV to infect transplanted cells (Li et al., 2015). Application of this technique to other cell-based therapies could soon follow. It will certainly be more challenging to edit cells *in vivo* but several groups are working toward this important goal, including developing strategies for cancer treatment (Sánchez-Rivera and Jacks, 2015).

TARGETING CELLS IN THE TME

Mononuclear Cells

Macrophages

Tumor-associated macrophages (TAMs) are often pro-tumoral by promoting angiogenesis, invasion, metastasis, and immune tolerance (Noy and Pollard, 2014). Given these and other important roles in tumor progression, there is a growing

interest in targeting TAMs as a cancer therapeutic. Macrophages are often characterized by the balance of pro- and anti-inflammatory characteristics as "M1" or "M2" macrophages, respectively. While in most cases this terminology is an oversimplification, it is a useful reference to describe macrophage subsets. M1 macrophages are characterized by high levels of major histocompatibility complex (MHC) class II molecules, pro-inflammatory cytokines and inducible nitric oxide synthase 2 (Nos2). Conversely, M2 macrophages express low levels of these markers and instead express high levels of arginase-1 and scavenger receptors like the mannose receptor (MR) (Martinez et al., 2009). Initial *in vitro* studies suggested macrophages could kill tumor cells, however, in the TME secreted factors shift TAMs to a pro-tumoral M2 phenotype (Sica et al., 2006). Therefore, reprograming TAMs to an M1-like state could reverse the pro-tumoral effects. We review here characteristics of NPs that promote macrophage uptake and detail studies utilizing NP gene delivery to target TAMs for cancer therapy.

Most NP platforms are in the size range of viruses (20–250 nm) and bacteria (0.2–10 μm), and consequently they are readily taken up by phagocytic cells of the immune system, such as macrophages and dendritic cells. In fact, uptake by the mononuclear phagocytic system (MPS), also called the reticuloendothelial system (RES), has been considered a critical obstacle to NP drug delivery. One example of this is the first FDA approved NP drug Doxil, a nanoliposome formulation of doxorubicin. Doxil was coated with a hydrophilic neutral polymer, PEG, to reduce its recognition by the MPS, creating a "stealth" effect (Working et al., 1994). Despite these modifications, clearance by macrophages still has a major influence on Doxil pharmacokinetics (La-Beck et al., 2012). The proclivity of macrophages for taking up NPs can even be utilized to image macrophages *in vivo* (Weissleder et al., 2014). Interestingly, M2 macrophages take up both 300 nm PEG hydrogel NPs and 30 nm quantum dots at higher rates than M1 macrophages due to increased levels of scavenger receptors such as MR, suggesting that TAMs may be especially sensitive to NP delivery. This phenomenon does not extend to microparticles, as 6 μm PEG hydrogels are not affected by macrophage polarization (Jones et al., 2013). Additionally, single cell pharmacokinetics of NPs within tumors has revealed that TAMs can serve as reservoirs for NPs, releasing small molecule drugs (Miller et al., 2015). The fate of oligonucleotide payloads in macrophages is less certain, although there is some evidence that macrophages may also transfer genes to cells at sites of inflammation (Haney et al., 2013; Mahajan et al., 2016). Because much effort has gone into reducing phagocyte clearance of NPs, there is a wealth of studies detailing the characteristics that reduce and consequently those that enhance uptake into this population. Detailed review of factors influencing macrophage NP uptake is covered elsewhere (Gustafson et al., 2015).

Macrophage NP uptake can occur through micropinocytosis, phagocytosis, and receptor-mediated endocytosis (Gustafson et al., 2015). Factors influencing uptake include charge, size, and surface chemistry. A positive surface charge facilitates uptake by many types of cells having a negative membrane potential. In contrast, greater net charge in either the positive or negative direction increases uptake of NPs by macrophages. The uptake of chitosan NPs with charges ranging from −40 to +35 was examined for murine macrophages. In these cells, increasing charge in both the negative and positive direction increased macrophage uptake, while non-phagocytic cells more efficiently took up positively charged particles. NP size is also a key determinant: large NPs 300–500 nm in size are taken up more efficiently than 150 nm particles by murine macrophages (He et al., 2010). In addition, uptake of particles by macrophages is highly dependent on serum protein adsorption. PEGylation can decrease, but not eliminate, protein adsorption and macrophage uptake (Xie et al., 2007; Walkey et al., 2012). These factors are important for non-targeted or passive uptake; however, further cell-type specificity can be achieved with targeting moieties.

Ligands or antibodies to cell surface receptors can be used to decorate the NP surface and enhance macrophage uptake. Receptors that mediate macrophage NP uptake include folate receptor (FR), MR, cluster of differentiation 163 (CD163), Legumanin, galactose-type C-type lectins, and cluster of differentiation 11b (CD11b) (Binnemars-Postma et al., 2017). Mannose is one of the most common macrophage targeting ligands, but MR (also known as CD206) is also present on other phagocytes, such as DCs (McKenzie et al., 2007), which could result in off-target effects. However, MR is upregulated in M2-like TAMs with decreased MHC II expression (Movahedi et al., 2010). Additionally, CCR2 knockout mice that have fewer TAMs have reduced tumor uptake of MR-targeted nanobodies, indicating that MR binding is through CCR2 derived cells, including macrophages (Movahedi et al., 2012). Mannosylated polymeric micelles are able to deliver siRNA and mediate TAM gene silencing *in vitro* and *in vivo* (Shann et al., 2013; Ortega et al., 2015). The galactose-type C lectin receptor has also been targeted for macrophage nucleic acid delivery by attaching its ligand, galactose, to the surface of NPs (Huang et al., 2012; Liu L. et al., 2017). One sophisticated approach utilized a dual targeting moiety: an apolipoprotein A1 mimetic (α-peptide) served as a ligand for SR-1B and was linked to a M2 macrophage binding protein (M2pep) to deliver NP-encapsulated siRNAs (Qian et al., 2017). Macrophages also express receptors capable of recognizing a variety of pathogen-associated molecular patterns (PAMPs) and incorporation of PAMPs into NP design can facilitate NP uptake by TAMs. For example, nanocomplexes incorporating glucan, a PAMP associated with fungi, were shown to target TAMs (Zhang et al., 2015a,b). In summary, TAMs act as natural sinks for NPs. Further targeting with receptor specific ligands or antibodies can facilitate uptake, but complete discrimination between macrophages and other mononuclear cells has not been clearly demonstrated.

A small number of studies have shown efficacy of nanoparticle nucleic acid delivery to target and reprogram TAMs for cancer therapy. In a melanoma mouse model, delivering anti-CSF-1R siRNA targeted to TAMs reduced tumor growth by 87% and prolonged survival. Non-targeted particles also inhibited tumor growth, but not as dramatically. This therapeutic effect corresponded with decreased immunosuppressive cytokines IL-10 and TGF-β, and increased immunostimmulatory cytokines IL-12 and IFN-γ as well as increased the function of CD8+

T-cells (Qian et al., 2017). Similarly, pro-inflammatory miR-155 was delivered in redox and pH sensitive NPs targeted with galactose moieties to the macrophage galactose-specific C-type lectin receptor. Galactose targeting increased *in vitro* miR-155 uptake in TAMs, but not B16-F10 cells. Delivery of miR-155 NPs increased IL-12 and MHCII positive cells, as well as decreased M2 markers. Increased numbers of activated T-cells and NK cells were observed, and anti-tumoral effects were elicited (Liu L. et al., 2017). Intratumoral injection of modified glucan nanocomplexes carrying siRNA has also been shown to effectively inhibit gene expression in macrophages. Delivery of siRNA to migration inhibitory factor (MIF) in glucan NPs reduced both released and intracellular MIF in TAMs and in cancer cells. This resulted in reduced M2 markers and increased inflammatory cytokines TNF-α and IL-2. This treatment also increased CD4+ and CD8+ cells in the tumor and promoted anti-tumor immunity (Zhang et al., 2015a,b). These reports support that oligonucleotide delivery can be used to reprogram TAMs from an M1 to M2 phenotype to promote anti-tumor effects.

Inflammatory Monocytes

Inflammatory monocytes (IMs) can give rise to TAMs and other myeloid suppressor cells which promote angiogenesis and subsequent metastasis. Recruitment of IMs relies on the chemokine CCL2 (Qian et al., 2011). Blocking this axis with receptors against CCL2 or its cognate receptor CCR2 has been the subject of clinical trials, but pharmacological inhibition of this axis has proved challenging in part due to rebound effects (Lim et al., 2016). An alternative strategy used screening approaches to identify both optimal lipids and siRNA sequences for inhibition of CCR2 in monocytes, no targeting ligands were used. In a lymphoma model, inhibition of CCR2 in monocytes reduced tumor size and number of TAMs. This therapy also inhibited expression of VEGF and reduced microvessel density in the tumors (Leuschner et al., 2011). Whether NP-based targeting of the CCL2-CCR2 axis can evade withdrawal effects seen with antibody targeting of the CCL2-CCR2 axis remains to be seen. However, the ability to silence genes in monocytes has demonstrated clear therapeutic potential.

Dendritic Cells

As part of the innate immune response to pathogens, dendritic cells recognize foreign materials through pattern recognition receptors (PRRs) or compliment binding leading to phagocytosis. Inside dendritic cell lysosomes, processing of pathogenic proteins results in the generation of peptide fragments that are presented on MHC receptors to be recognized by members of the adaptive immune system. Cross presentation of antigens from DCs to $CD8^+$ T-cells is required for anti-tumor immunity. As such, DCs are the primary targets of cancer vaccines and their actions are required for effective cytotoxic T-cell response in checkpoint blockade inhibitor therapies. NPs from 20 nm to 3 μm are readily taken up by dendritic cells, presumably due to the size similarity to viral and bacterial pathogens. The size of NPs and their ability to present multivalent antigens clearly points to vaccine applications (Bachmann and Jennings, 2010). For these reasons an increasing number of NP-based vaccines with or without additional immune agonists are being designed for cancer therapy (Mizrahy et al., 2017). Here we will consider those that additionally incorporate nucleic acid delivery. A smaller number of studies have utilized gene delivery to modulate the activation of dendritic cells and subsequent cross-presentation to T-cells.

Surface coating can also increase DC NP uptake. For example, natural coatings such as mannosylation or glycosylation increase DC uptake through interactions with MR (Jiang et al., 2015; De Coen et al., 2016; Wang et al., 2016). However, as previously described, MRs are also expressed on macrophages which may compete with DCs for NP uptake (Stahl and Ezekowitz, 1998). Alternatively, antibody-based targeting has also been reported. One example is a clinical trial using targeting antibodies against MR to deliver a peptide antigen to APCs. This therapy induced humoral and T-cell responses in melanoma patients (Morse et al., 2011). Reports indicate that antibodies against DEC-205 can also enhance DC uptake and increase downstream immune activation relative to non-targeted NPs (Raghuwanshi et al., 2012; Walters et al., 2015). Using antibodies against DEC-205 fused with a tumor antigen induced humoral and cellular immunity in patients with advanced malignancies (Dhodapkar et al., 2014). While MR and DEC-205 are commonly used for NP or vaccine targeting, other targets have also been tested (Sehgal et al., 2014a), including targets that enhance uptake in subsets of DCs (Sehgal et al., 2014b). However, in some cases the material composition of the NP carrier may be more important than targeting ligands. For instance, lipid based "nanogels" were more readily internalized than PLGA NPs (Look et al., 2014). Also, linear PEI nanocomplexes were more effective than anti-CD11c antibody-targeted zwitterionic liposomes at siRNA delivery to DCs (Cubillos-Ruiz et al., 2009).

Several reports both *in vitro* and *in vivo* have shown that delivering siRNAs in addition to antigenic peptides and adjuvants to DCs can further enhance anti-tumor immune responses. Heo et al. (2014) used polymeric micelles to deliver a tumor antigen and siRNA for the immunosuppressive Suppressor of Cytokine Signaling 1 (SOCS1) to dendritic cells *in vitro*. Delivery of SOCS1 siRNA increased secretion of pro-inflammatory cytokines by cultured DCs and activation of T-cells by cross presentation (Heo et al., 2014). Two additional studies by Heo et al. (2014) examined *in vivo* efficacy of NP siRNA to DCs. In one study, the investigators formulated multifunctional polymeric NPs carrying tumor model antigen OVA, dendritic cell activator imiquimod (R837), and siRNAs for STAT3. The immune activation induced by R837 is inhibited by STAT3, so the authors hypothesized that this combination would produce a more robust activation of DCs. PLGA (R837/STAT3 siRNA) NPs were taken up efficiently by DCs, elicited cytokine response, antigen cross-presentation, and trafficking of DCs to draining lymph nodes when injected *in vivo*. Furthermore, incorporation of STAT3 siRNA significantly increased anti-tumor immunity (Heo and Lim, 2014). In a different approach, tumor bearing mice were first treated with hyaluronic acid (HA) and paclitaxel (PTX) complexes to induce immunogenic cell death. This treatment was followed by administration of NPs containing CpG adjuvant and IL-10 siRNAs. IL-10 is an immunosuppressive cytokine and

its inhibition further enhanced the immune response. These multifunctional NPs trafficked to draining LNs and promoted antitumor immunity *in vivo* (Heo et al., 2015).

In ovarian cancer tumor-associated DCs (tDCs) have a particularly tolerogenic role (Huarte et al., 2008). By inhibiting tolerogenic pathways in DCs with siRNA, therapeutic benefits were observed in ovarian cancer models. In addition to siRNA, miRNA can also be delivered to tDCs to induce anti-tumor immunity. For example, delivery of siRNAs against members of the ER stress pathway, XBP1 and IRE1, which inhibit cross presentation in tDC through lipid accumulation. NP delivery of XBP1 or IRE1 siRNA reduced metastasis and increased survival in ovarian cancer models. Importantly, this phenomenon was ablated in Rag2 deficient mice suggesting that immune and not direct cancer targets were responsible (Cubillos-Ruiz et al., 2015). miR-155 is considered to be an oncogenic miRNA, however, it is also necessary for cross-presentation of DCs. Using PEI NPs, delivery of miR-155 mimics produced potent anti-tumor effects with about 33% of mice showing no disease progression 80 days after controls had succumbed to disease. This anti-tumor effect was accompanied by transcriptome wide changes in tDCs, highlighting the utility of miRNAs for reprograming the TME (Cubillos-Ruiz et al., 2012). Interestingly, the authors also found that a bulged dsRNA that required processing by RNase enzyme DICER was most effective at gene silencing. Another aspect of the studies by Cubillos-Ruiz et al. (2009, 2012, 2015) was an immune-stimulatory effect of PEI NPs containing even non-targeting RNA through TLR pathways. Overall, RNA delivery to tDCs has been shown to be an effective therapeutic strategy in mouse models of ovarian cancer.

Historically, vaccines have relied on peptide antigens, but an alternative vaccine strategy is delivery of DNA or mRNAs. In brief, DNA or mRNA encoding an antigen are injected, the genetic material is taken up by cells at the injection site, and then translated into protein. Proteins encoded in the DNA or mRNA can be expressed in myocytes or keratinocytes at the injection site and are subsequently recognized by APCs or directly taken up by DCs followed by internal processing and presentation. DNA vaccines are currently used in veterinary medicine, but have thus far not been successfully translated to humans (Rice et al., 2008). Attempts at DNA vaccines in humans have relied on non-specific targeting of injected DNA. Increased gene delivery through electroporation and NP delivery systems has been reported, but generally do not utilize cell-specific targeting. In one report, plasmid DNA for the nucleocapsid of severe acute respiratory syndrome coronavirus (SARS-CoV) was delivered in chitosan NPs targeted with anti-DEC-205 antibody. NP DNA delivery successfully stimulated IgG and IgA antibodies against SARS-CoV nucleocapsid, in contrast to naked DNA, which produced no detectable antibody response. In addition, DC targeting with anti-DEC-205 antibody significantly increased serum IgG against SARS-CoV nucleocapsid (Raghuwanshi et al., 2012). This approach could be translated to cancer immunotherapy as well, but consideration of particle size may be critical to induce a cytotoxic T-cell (CTL) response, given that $CD8^+$ DCs are necessary to induce a CTL response and are restricted to the lymph node. Therefore, particles must drain to the lymph node, which requires a particle size of 20–200, with 40 nm being ideal (Bachmann and Jennings, 2010).

Overall, DCs are an exciting TME target for NP nucleic acid delivery; they are intimately involved in the anti-tumor response and are required for the actions of checkpoint blockade therapies. Serendipitously, their phagocytic abilities and PRRs also make them easy targets for NP delivery. These qualities have generated increased interest in NP-based vaccines, which will likely lead to several clinical trials. As multifunctional NPs are designed to deliver antigens and adjuvants to DCs, gene delivery strategies should also be considered.

Cancer-Associated Fibroblasts

Within the tumor stroma, cancer-associated fibroblasts (CAFs) modulate tumor growth and metastasis by secreting growth factors, chemokines, and extracellular components (Kalluri and Zeisberg, 2006). In many tumors, especially desmoplastic tumors with a dense stroma, CAFs often lie between blood vessels and cancer cells. This makes CAFs an impediment to cancer-directed NP delivery (Miao et al., 2016). Cisplatin NPs with or without targeting are largely taken up by CAFs in desmoplastic pancreatic tumors (Miao et al., 2017b). Damage to fibroblasts initially reduces their supportive role and promotes tumor regression. However, chronic exposure induces expression and release of soluble factors such as Wnt16 and resistance to chemotherapy. Co-delivering Wnt16 siRNA along with cisplatin NPs can prevent resistance through this pathway (Miao et al., 2015). Since this finding, several studies have now shown that plasmid DNA can be delivered to and expressed in CAFs using lipid-based NPs. In one study, delivery of a gene that produced a soluble TNFα-related apoptosis inducing ligand (sTRAIL) to CAFs caused apoptosis in the tumor parenchyma, and ultimately tumor regression (Miao et al., 2017b). Similarly, several studies have shown that delivery of pDNA encoding "traps" can be successfully delivered to CAFs *in vivo* for cancer therapy. Traps are fusion proteins designed to be secreted and ultimately bound to soluble factors in the TME, such as chemokines and cytokines. By inhibiting these factors, metastasis and immunosuppression have been shown to be reduced, ultimately improving survival in animal models. In one report, a CXCL12 trap in combination with a PD-L1 trap promoted T-cell infiltration and reduced liver metastasis of pancreatic cancer more than either therapy alone (Miao et al., 2017a). Combination of CXCL12 and PD-L1 traps also decreased immune suppressive lymphoid structures and enhanced anti-cancer vaccine efficacy (Goodwin et al., 2017). These studies together suggest that CAFs can be used as cellular factories for production of proteins that inhibit the immunosuppressive TME. This work demonstrates the possibility that replacement or inhibition of endogenous genes in CAFs may be a feasible therapeutic strategy.

T-cells

T-cells are important mediators of anti-tumor immunity and the targets of immune-oncology drugs such as checkpoint blockade inhibitors. The receptors PD-1 and CTLA4 expressed on T-cells promote exhaustion and thus immune evasion by cancer cells. Antibodies blocking these receptors and their

ligands have proven to be effective stimulators of anti-tumor immunity and have quickly become a staple of anti-cancer therapy (Hoos, 2016). An alternative T-cell based approach to promote immune recognition of cancer cells is autologous, genetically engineered T-cells. These cells are engineered to express CARs specific to cancer epitopes using viral transduction. CAR-T cells have recently been approved by the FDA for acute lymphoblastic leukemia (ALL) and clinical trials are ongoing in many other cancer types (Landoni and Savoldo, 2017). Whether by removing checkpoint blockade or through genetic modification, T-cells have been demonstrated to be a successful target for cancer immunotherapy. New approaches to further harness the power of T-cells are being developed in many areas, including nanomedicine.

CAR-T-cells

Expression of CARs in primary T-cells relies on viral transduction and integration of DNA into the genome *in vitro*. Manufacturing genetically engineered cells for autologous transplantation is an intensive process with relatively low yields. T-cells are resistant to many forms of gene delivery and standard transfection protocols are not effective. Current gene delivery methods to T-cells rely on viruses or electroporation (Freeley and Long, 2013). Viral methods can be mutagenic and electroporation of cell membranes can lead to irreversible cell damage and low yields. The incorporation of efficient and transient gene expression with NP platforms to produce engineered T-cells holds promise for improved immunotherapies. Photoporation based on NPs is one such strategy. In this approach, transient permeabilization is achieved by adding gold NPs to CD8+ T-cells followed by short laser pulses, creating a photothermal effect. This strategy had lower cytotoxicity than nucleofection with comparable siRNA-mediated gene knockdown (Wayteck et al., 2017).

One potential improvement to CAR T-cell therapy is increasing the specificity of T-cells by means of removing non-cancer specific TCRs. Toward this aim, Moffett et al. report NP delivery of mRNAs to T-cells using anti-CD3 and anti-CD8 antibody targeting. Delivery of megaTAL nuclease mediated elimination of the T-cell receptor alpha constant region (TRAC), effectively removed the ability of T-cells to produce their own TCRs and resulted in the specific expression of the CAR (Moffett et al., 2017). This approach may foreseeably reduce off-target immune responses, but was not tested *in vivo*. In another approach, Moffett et al. (2017) increased the proportion of central memory T-cells, a critical cell population in establishing an effective immune response. Enrichment of the central memory T-cell phenotype was achieved by introducing $Foxo1_{3A}$ encoding mRNA into a CD3 targeted NP platform. Treatment of T-cells with these $Foxo1_{3A}$-encoding NPs increased the activity of CAR-modified T-cells in a mouse model of B-cell lymphoma (Moffett et al., 2017).

While these reports are intriguing, given the prevalence of viral methods in autologous T-cell therapy, it is questionable whether NP-based T-cell gene delivery will be clinically translatable. Despite greater than 200 clinical trials for CAR-T cells, none currently use NP-based methods.

T-cells *in Vivo*

Nanoparticle-mediated nucleic acid delivery to T-cells *in vivo* has also been demonstrated. These NP systems rely on antibodies to surface proteins expressed on T-cells. In one instance, b7 integrin targeting antibody was used to deliver lipid-based NPs containing siRNAs to leukocytes. Systemic delivery of only 2.5 mg/kg mediated gene knockdown (Peer et al., 2008). Delivery of siRNAs to CCR5, a critical receptor for HIV entry, with lymphocyte function-associated antigen 1 (LFA-1)-targeted particles decreased susceptibility of humanized mice to HIV infection (Kim et al., 2010). In these two studies, the subsets of leukocytes targeted were not described and are likely heterogeneous, considering the targeted receptors are present on many leukocytes. Alternatively, using anti-CD4 antibody decorated lipid NPs can specifically deliver siRNA to T-cells *in vivo*. Ramishetti et al. (2015) found that internalization, not endosomal escape, may be the limiting factor for T-cell gene delivery. Intriguingly, CD4 subsets with high or low CD4 expression had different rates of internalization and subsequent gene silencing (Ramishetti et al., 2015). Further research into the T-cell internalization pathways and characterization of internalization after binding to other T-cell specific receptors is warranted. Collectively, *in vivo* delivery of oligonucleotides to T-cells with NPs is achievable, but the potential therapeutic benefit for cancer is yet to be determined.

Blood Vessels

Angiogenesis refers to the growth of new blood vessels from pre-existing vascular networks. Healthy vasculature is quiescent due to a controlled balance between pro- (e.g., VEGF and FGF) and anti- (e.g., angiostatin and thrombospondin) angiogenic factors that regulate endothelial cell proliferation and migration (Jain, 2003). As tumors outgrow their local oxygen supply, they hijack this regulation and permanently shift the balance to a pathologic, pro-angiogenic state during the "angiogenic switch" (Folkman, 1971; Hanahan and Folkman, 1996). This produces chaotic and dysfunctional vasculature. While normal blood vessels consist of a continuous monolayer of tightly adhered ECs, closely associated mural cells that promote vessel stability, and a continuous basement membrane; tumor vessels have loosely associated ECs with large gaps between them, poor mural cell recruitment, and an irregular and discontinuous basement membrane (Baluk et al., 2005). This reduced vessel wall integrity promotes leakiness and cancer cell intravasation. Thus, directly targeting tumor vessels to either inhibit their growth or promote their normalization is believed to have the potential to inhibit tumor growth and aggression, as well as metastasis (Folkman, 1971; Carmeliet and Jain, 2011). Interestingly, it is the "leaky" nature of tumor blood vessels that both makes it challenging to deliver drugs such as chemotherapy to the tumor core, but also greatly facilitates delivery of NPs to cancer cells due to the "enhanced permeability and retention effect" (Prabhakar et al., 2013).

Oligonucleotide delivery to tumor endothelium has been achieved with multiple NP platforms. Generally, successful delivery of NPs to vasculature is confirmed by visualizing co-localization of fluorescently labeled nucleic acids packaged in

NPs with an endothelial stain such as the cell surface marker CD31. Chitosan NPs have been demonstrated to co-localize to both tumor and endothelial cells *in vivo* and effectively deliver siRNAs to both cell types (Lu et al., 2010). In an orthotopic model of ovarian carcinoma, treatment with chitosan NPs carrying siRNAs targeting human EZH2 (expressed in the transplanted cancer cells) or murine EZH2 (expressed in the endogenous murine vasculature) inhibited tumor growth. However, the NPs carrying murine targeting siRNA had more potent effects on inhibiting disease burden, suggesting chitosan-mediated targeting of tumor vasculature had more potent therapeutic effects than targeting cancer cells directly (Lu et al., 2010). Second-generation NPs rely on incorporation of ligands to target endothelial cell-specific surface proteins. For example, ligands to integrin αVβ3, such as the peptide RGD, can be used to facilitate NP uptake into neo-vasculature. Studies have shown that NPs containing the chemotherapeutic drug doxorubicin can be directed specifically to tumor vasculature using this ligand, causing loss of tumor blood vessels and decreased metastasis (Murphy et al., 2008). Similarly, delivery of an anti-miR to inhibit the pro-angiogenic miR-132 with these same NPs in an orthotopic xenograft mouse model of human breast cancer yielded therapeutic effects on inhibiting tumor vasculature and decreasing tumor burden (Anand et al., 2010). miRNAs have also been delivered using RGD-labeled chitosan NPs. Delivery of miR-200 family members using this approach reduced angiogenesis by direct and indirect mechanisms and resulted in reduced disease burden in ovarian cancer models (Pecot et al., 2013). RGD-chitosan mediated delivery of siRNA targeting *PLXDC1*, a growth-promoting gene, has been shown to effectively silence target gene expression in endothelial cells, with subsequent effects on promoting endothelial apoptosis and inhibiting tumor growth (Hee-Dong et al., 2010). The $\alpha_V\beta_3$ integrin also facilitates uptake of viral genomic material and therefore may be an effective route for NP based gene delivery (Stewart and Nemerow, 2007). In one report, delivery of mutant *Raf-1* gene with $\alpha_V\beta_3$-targeted cationic lipid NPs caused apoptosis of vessels and surrounding tumor tissues (Hood et al., 2002). Another receptor that can mediate uptake into the vascular endothelium is CD31, a classical marker of blood vessels. While αVβ3 is thought to be expressed specifically by tumor neovasculature (as well as some cancer cell types), CD31 is expressed on all endothelium (both blood, and to a lesser extent, lymphatic). Using CD31 ligands to deliver siRNAs resulted in specific decrease of target genes in vascular endothelium. By delivering siRNA to CD31 itself, tumor growth and metastasis were inhibited in a prostate cancer model (Santel et al., 2006). An alternative approach to ligand-based targeting is chemically modified dendrimers that can specifically target the endothelium (Khan et al., 2015). 7C1 NPs are another type of NP that have been reported to localize faithfully and specifically to the endothelium in multiple models of aberrant vascular function, including tumor angiogenesis. These NPs are able to elicit at least 50% knockdown of target endothelial gene expression, and simultaneously deliver siRNAs targeting multiple genes in the endothelium (Dahlman et al., 2014).

SUMMARY

In an era where clinical trials in nucleic acid delivery have become a reality, we can expand our scope to consider new and exciting gene and cell targets for cancer therapy. NP uptake by cells within the TME has traditionally been considered a delivery obstacle for NP-based systems, however, turning TME cells into targets could lead to new therapeutic strategies. Biology has taught us that non-transformed cells can act as accessories to cancer growth and spread, but that strategies to reprogram cells in the TME could result in revolutionary therapies. The studies highlighted in this review demonstrate NP-based nucleic acid delivery strategies for reprograming the TME. In effect, turning the TME from a permissive space for cancer growth to a hostile one. This strategy is synergistic with current immunotherapy and anti-angiogenic approaches and could feasibly extend the efficacy of these paradigm-shifting treatments.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Anand, S., Majeti, B. K., Acevedo, L. M., Murphy, E. A., Mukthavaram, R., Scheppke, L., et al. (2010). MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. *Nat. Med.* 16, 909–914. doi: 10.1038/nm.2186

Anwer, K., Barnes, M., Fewell, J., Lewis, D., and Alvarez, R. (2010). Phase-I clinical trial of IL-12 plasmid/lipopolymer complexes for the treatment of recurrent ovarian cancer. *Gene Ther.* 17, 360–369. doi: 10.1038/gt.2009.159

Bachmann, M. F., and Jennings, G. T. (2010). Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat. Rev. Immunol.* 10, 787–796. doi: 10.1038/nri2868

Baluk, P., Hashizume, H., and McDonald, D. M. (2005). Cellular abnormalities of blood vessels as targets in cancer. *Curr. Opin. Genet. Dev.* 15, 102–111. doi: 10.1016/j.gde.2004.12.005

Baum, C., Kustikova, O., Modlich, U., Li, Z., and Fehse, B. (2006). Mutagenesis and oncogenesis by chromosomal insertion of gene transfer vectors. *Hum. Gene Ther.* 17, 253–263. doi: 10.1089/hum.2006.17.253

Beg, M. S., Brenner, A. J., Sachdev, J., Borad, M., Kang, Y.-K., Stoudemire, J., et al. (2017). Phase I study of MRX34, a liposomal miR-34a mimic, administered twice weekly in patients with advanced solid tumors. *Invest. New Drugs* 35, 180–188. doi: 10.1007/s10637-016-0407-y

Binnemars-Postma, K., Storm, G., and Prakash, J. (2017). Nanomedicine strategies to target tumor-associated macrophages. *Int. J. Mol. Sci.* 18:E979. doi: 10.3390/ijms18050979

Bivas-Benita, M., Romeijn, S., Junginger, H. E., and Borchard, G. (2004). PLGA–PEI nanoparticles for gene delivery to pulmonary epithelium. *Eur. J. Pharm. Biopharm.* 58, 1–6. doi: 10.1016/j.ejpb.2004.03.008

Brandén, L. J., Mohamed, A. J., and Smith, C. E. (1999). A peptide nucleic acid–nuclear localization signal fusion that mediates nuclear transport of DNA. *Nat. Biotechnol.* 17, 784–787.

Buscail, L., Bournet, B., Vernejoul, F., Cambois, G., Lulka, H., Hanoun, N., et al. (2015). First-in-man phase 1 clinical trial of gene therapy for advanced pancreatic cancer: safety, biodistribution, and preliminary clinical findings. *Mol. Ther.* 23, 779–789. doi: 10.1038/mt.2015.1

Capecchi, M. R. (1980). High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* 22, 479–488. doi: 10.1016/0092-8674(80)90358-X

Carmeliet, P., and Jain, R. K. (2011). Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat. Rev. Drug Discov.* 10, 417–427. doi: 10.1038/nrd3455

Chang, J. Y., Xia, W., Shao, R., Sorgi, F., Hortobagyi, G. N., Huang, L., et al. (1997). The tumor suppression activity of E1A in HER-2/neu-overexpressing breast cancer. *Oncogene* 14, 561–568. doi: 10.1038/sj.onc.1200861

Chou, J., Shahi, P., and Werb, Z. (2013). microRNA-mediated regulation of the tumor microenvironment. *Cell Cycle* 12, 3262–3271. doi: 10.4161/cc.26087

Cox, D. B. T., Platt, R. J., and Zhang, F. (2015). Therapeutic genome editing: prospects and challenges. *Nat. Med.* 21, 121–131. doi: 10.1038/nm.3793

Cubillos-Ruiz, J. R., Baird, J. R., Tesone, A. J., Rutkowski, M. R., Scarlett, U. K., Camposeco-Jacobs, A. L., et al. (2012). Reprogramming tumor-associated dendritic cells in vivo using miRNA mimetics triggers protective immunity against ovarian cancer. *Cancer Res.* 72, 1683–1693. doi: 10.1158/0008-5472.CAN-11-3160

Cubillos-Ruiz, J. R., Engle, X., Scarlett, U. K., Martinez, D., Barber, A., Elgueta, R., et al. (2009). Polyethylenimine-based siRNA nanocomplexes reprogram tumor-associated dendritic cells via TLR5 to elicit therapeutic antitumor immunity. *J. Clin. Invest.* 119, 2231–2244. doi: 10.1172/JCI37716

Cubillos-Ruiz, J. R., Silberman, P. C., Rutkowski, M. R., Chopra, S., Perales-Puchalt, A., Song, M., et al. (2015). ER stress sensor XBP1 controls anti-tumor immunity by disrupting dendritic cell homeostasis. *Cell* 161, 1527–1538. doi: 10.1016/j.cell.2015.05.025

Cyranoski, D. (2016). CRISPR gene-editing tested in a person for the first time. *Nat. News* 539:479. doi: 10.1038/nature.2016.20988

Dahlman, J. E., Barnes, C., Khan, O. F., Thiriot, A., Jhunjunwala, S., Shaw, T. E., et al. (2014). In vivo endothelial siRNA delivery using polymeric nanoparticles with low molecular weight. *Nat. Nanotechnol.* 9, 648–655. doi: 10.1038/nnano.2014.84

De Coen, R., Vanparijs, N., Risseeuw, M. D., Lybaert, L., Louage, B., De Koker, S., et al. (2016). pH-degradable mannosylated nanogels for dendritic cell targeting. *Biomacromolecules* 17, 2479–2488. doi: 10.1021/acs.biomac.6b00685

Dhodapkar, M. V., Sznol, M., Zhao, B., Wang, D., Carvajal, R. D., Keohan, M. L., et al. (2014). Induction of antigen-specific immunity with a vaccine targeting NY-ESO-1 to the dendritic cell receptor DEC-205. *Sci. Transl. Med.* 6, ra251–ra232. doi: 10.1126/scitranslmed.3008068

Esteller, M. (2011). Non-coding RNAs in human disease. *Nat. Rev. Genet.* 12, 861–874. doi: 10.1038/nrg3074

Farazi, T. A., Hoell, J. I., Morozov, P., and Tuschl, T. (2013). MicroRNAs in human cancer. *Adv. Exp. Med. Biol.* 774, 1–20. doi: 10.1007/978-94-007-5590-1_1

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., et al. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.* 84, 7413–7417. doi: 10.1073/pnas.84.21.7413

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811.

Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* 285, 1182–1186. doi: 10.1056/NEJM197111182852108

Freeley, M., and Long, A. (2013). Advances in siRNA delivery to T-cells: potential clinical applications for inflammatory disease, cancer and infection. *Biochem. J.* 455, 133–147. doi: 10.1042/BJ20130950

Gallie, D. R. (1991). The cap and poly (A) tail function synergistically to regulate mRNA translational efficiency. *Genes Dev.* 5, 2108–2116. doi: 10.1101/gad.5.11.2108

Glover, D. J., Lipps, H. J., and Jans, D. A. (2005). Towards safe, non-viral therapeutic gene expression in humans. *Nat. Rev. Genet.* 6, 299–311. doi: 10.1038/nrg1577

Gofrit, O. N., Benjamin, S., Halachmi, S., Leibovitch, I., Dotan, Z., Lamm, D. L., et al. (2014). DNA based therapy with diphtheria toxin-A BC-819: a phase 2b marker lesion trial in patients with intermediate risk nonmuscle invasive bladder cancer. *J. Urol.* 191, 1697–1702. doi: 10.1016/j.juro.2013.12.011

Goodwin, T. J., Shen, L., Hu, M., Li, J., Feng, R., Dorosheva, O., et al. (2017). Liver specific gene immunotherapies resolve immune suppressive ectopic lymphoid structures of liver metastases and prolong survival. *Biomaterials* 141, 260–271. doi: 10.1016/j.biomaterials.2017.07.007

Gorski, K., Carneiro, M., and Schibler, U. (1986). Tissue-specific in vitro transcription from the mouse albumin promoter. *Cell* 47, 767–776. doi: 10.1016/0092-8674(86)90519-2

Gurunathan, S., Klinman, D. M., and Seder, R. A. (2000). DNA vaccines: immunology, application, and optimization. *Annu. Rev. Immunol.* 18, 927–974. doi: 10.1146/annurev.immunol.18.1.927

Gustafson, H. H., Holt-Casper, D., Grainger, D. W., and Ghandehari, H. (2015). Nanoparticle uptake: the phagocyte problem. *Nano Today* 10, 487–510. doi: 10.1016/j.nantod.2015.06.006

Gutschner, T., and Diederichs, S. (2012). The hallmarks of cancer: a long non-coding RNA point of view. *RNA Biol.* 9, 703–719. doi: 10.4161/rna.20481

Ha, M., and Kim, V. N. (2014). Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol.* 15, 509–524. doi: 10.1038/nrm3838

Hanahan, D., and Coussens, L. M. (2012). Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 21, 309–322. doi: 10.1016/j.ccr.2012.02.022

Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353–364. doi: 10.1016/S0092-8674(00)80108-7

Haney, M. J., Zhao, Y., Harrison, E. B., Mahajan, V., Ahmed, S., He, Z., et al. (2013). Specific transfection of inflamed brain by macrophages: a new therapeutic strategy for neurodegenerative diseases. *PLoS One* 8:e61852. doi: 10.1371/journal.pone.0061852

Hanna, N., Ohana, P., Konikoff, F., Leichtmann, G., Hubert, A., Appelbaum, L., et al. (2012). Phase 1/2a, dose-escalation, safety, pharmacokinetic and preliminary efficacy study of intratumoral administration of BC-819 in patients with unresectable pancreatic cancer. *Cancer Gene Ther.* 19, 374–382. doi: 10.1038/cgt.2012.10

He, C., Hu, Y., Yin, L., Tang, C., and Yin, C. (2010). Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. *Biomaterials* 31, 3657–3666. doi: 10.1016/j.biomaterials.2010.01.065

Hee-Dong, H., Mangala, L. S., Lee, J. W., Shahzad, M. M., Kim, H.-S., Shen, D.-Y., et al. (2010). Targeted gene silencing using RGD-labeled chitosan nanoparticles. *Clin. Cancer Res.* 16, 3910–3922. doi: 10.1158/1078-0432.CCR-10-0005

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., et al. (2000). A Toll-like receptor recognizes bacterial DNA. *Nature* 408, 740–745. doi: 10.1038/35047123

Heo, M. B., Cho, M. Y., and Lim, Y. T. (2014). Polymer nanoparticles for enhanced immune response: combined delivery of tumor antigen and small interference RNA for immunosuppressive gene to dendritic cells. *Acta Biomater.* 10, 2169–2176. doi: 10.1016/j.actbio.2013.12.050

Heo, M. B., Kim, S.-Y., Yun, W. S., and Lim, Y. T. (2015). Sequential delivery of an anticancer drug and combined immunomodulatory nanoparticles for efficient chemoimmunotherapy. *Int. J. Nanomed.* 10, 5981–5992. doi: 10.2147/IJN.S90104

Heo, M. B., and Lim, Y. T. (2014). Programmed nanoparticles for combined immunomodulation, antigen presentation and tracking of immunotherapeutic cells. *Biomaterials* 35, 590–600. doi: 10.1016/j.biomaterials.2013.10.009

Holtkamp, S., Kreiter, S., Selmi, A., Simon, P., Koslowski, M., Huber, C., et al. (2006). Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. *Blood* 108, 4009–4017. doi: 10.1182/blood-2006-04-015024

Hood, J. D., Bednarski, M., Frausto, R., Guccione, S., Reisfeld, R. A., Xiang, R., et al. (2002). Tumor regression by targeted gene delivery to the neovasculature. *Science* 296, 2404–2407. doi: 10.1126/science.1070200

Hoos, A. (2016). Development of immuno-oncology drugs [mdash] from CTLA4 to PD1 to the next generations. *Nat. Rev. Drug Discov.* 15, 235–247. doi: 10.1038/nrd.2015.35

Huang, Z., Zhang, Z., Jiang, Y., Zhang, D., Chen, J., Dong, L., et al. (2012). Targeted delivery of oligonucleotides into tumor-associated macrophages for cancer immunotherapy. *J. Control. Release* 158, 286–292. doi: 10.1016/j.jconrel.2011.11.013

Huarte, E., Cubillos-Ruiz, J. R., Nesbeth, Y. C., Scarlett, U. K., Martinez, D. G., Buckanovich, R. J., et al. (2008). Depletion of dendritic cells delays ovarian cancer progression by boosting antitumor immunity. *Cancer Res.* 68, 7684–7691. doi: 10.1158/0008-5472.CAN-08-1167

Hutvágner, G., McLachlan, J., Pasquinelli, A. E., Bálint, É., Tuschl, T., and Zamore, P. D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* 293, 834–838. doi: 10.1126/science.1062961

Jain, R. K. (2003). Molecular regulation of vessel maturation. *Nat. Med.* 9, 685–693. doi: 10.1038/nm0603-685

Jiang, P.-L., Lin, H.-J., Wang, H.-W., Tsai, W.-Y., Lin, S.-F., Chien, M.-Y., et al. (2015). Galactosylated liposome as a dendritic cell-targeted mucosal vaccine for inducing protective anti-tumor immunity. *Acta Biomater.* 11, 356–367. doi: 10.1016/j.actbio.2014.09.019

Jones, S. W., Roberts, R. A., Robbins, G. R., Perry, J. L., Kai, M. P., Chen, K., et al. (2013). Nanoparticle clearance is governed by Th1/Th2 immunity and strain background. *J. Clin. Invest.* 123, 3061–3073. doi: 10.1172/JCI66895

Judge, A. D., Bola, G., Lee, A. C., and MacLachlan, I. (2006). Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. *Mol. Ther.* 13, 494–505. doi: 10.1016/j.ymthe.2005.11.002

Judge, A. D., Sood, V., Shaw, J. R., Fang, D., McClintock, K., and MacLachlan, I. (2005). Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat. Biotechnol.* 23, 457–462. doi: 10.1038/nbt1081

Kaczmarek, J. C., Kowalski, P. S., and Anderson, D. G. (2017). Advances in the delivery of RNA therapeutics: from concept to clinical reality. *Genome Med.* 9:60. doi: 10.1186/s13073-017-0450-0

Kalluri, R., and Zeisberg, M. (2006). Fibroblasts in cancer. *Nat. Rev. Cancer* 6, 392–401. doi: 10.1038/nrc1877

Karikó, K., Buckstein, M., Ni, H., and Weissman, D. (2005). Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* 23, 165–175. doi: 10.1016/j.immuni.2005.06.008

Kendrick, J., Matthews, K., Straughn, J. Jr., Barnes, M., Fewell, J., Anwer, K., et al. (2008). A phase I trial of intraperitoneal EGEN-001, a novel IL-12 gene therapeutic, administered alone or in combination with chemotherapy in patients with recurrent ovarian cancer. *J. Clin. Oncol.* 26, 5572–5572. doi: 10.1200/jco.2008.26.15_suppl.5572

Khan, O. F., Zaia, E. W., Jhunjhunwala, S., Xue, W., Cai, W., Yun, D. S., et al. (2015). Dendrimer-inspired nanomaterials for the *in vivo* delivery of siRNA to lung vasculature. *Nano Lett.* 15, 3008–3016. doi: 10.1021/nl5048972

Kim, S.-S., Peer, D., Kumar, P., Subramanya, S., Wu, H., Asthana, D., et al. (2010). RNAi-mediated CCR5 silencing by LFA-1-targeted nanoparticles prevents HIV infection in BLT mice. *Mol. Ther.* 18, 370–376. doi: 10.1038/mt.2009.271

Klinman, D. M., Yi, A.-K., Beaucage, S. L., Conover, J., and Krieg, A. M. (1996). CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. U.S.A.* 93, 2879–2883. doi: 10.1073/pnas.93.7.2879

Kosik, K. S. (2010). MicroRNAs and cellular phenotypy. *Cell* 143, 21–26. doi: 10.1016/j.cell.2010.09.008

Krieg, A. M., Wu, T., Weeratna, R., Efler, S. M., Love-Homan, L., Yang, L., et al. (1998). Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc. Natl. Acad. Sci. U.S.A.* 95, 12631–12636. doi: 10.1073/pnas.95.21.12631

Krieg, A. M., Yi, A.-K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., et al. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374, 546–549. doi: 10.1038/374546a0

La-Beck, N. M., Zamboni, B. A., Gabizon, A., Schmeeda, H., Amantea, M., Gehrig, P. A., et al. (2012). Factors affecting the pharmacokinetics of pegylated liposomal doxorubicin in patients. *Cancer Chemother. Pharmacol.* 69, 43–50. doi: 10.1007/s00280-011-1664-2

Landoni, E., and Savoldo, B. (2017). Treating hematological malignancies with cell therapy: where are we now? *Expert Opin. Biol. Ther.* 18, 65–75. doi: 10.1080/14712598.2018.1384810

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425, 415–419. doi: 10.1038/nature01957

Leuschner, F., Dutta, P., Gorbatov, R., Novobrantseva, T. I., Donahoe, J. S., Courties, G., et al. (2011). Therapeutic siRNA silencing in inflammatory monocytes in mice. *Nat. Biotechnol.* 29, 1005–1010. doi: 10.1038/nbt.1989

Li, C., Guan, X., Du, T., Jin, W., Wu, B., Liu, Y., et al. (2015). Inhibition of HIV-1 infection of primary CD4+ T-cells by gene editing of CCR5 using adenovirus-delivered CRISPR/Cas9. *J. Gen. Virol.* 96, 2381–2393. doi: 10.1099/vir.0.000139

Lim, S. Y., Yuzhalin, A. E., Gordon-Weeks, A. N., and Muschel, R. J. (2016). Targeting the CCL2-CCR2 signaling axis in cancer metastasis. *Oncotarget* 7, 28697–28710. doi: 10.18632/oncotarget.7376

Ling, H., Fabbri, M., and Calin, G. A. (2013). MicroRNAs and other non-coding RNAs as targets for anticancer drug development. *Nat. Rev. Drug Discov.* 12, 847–865. doi: 10.1038/nrd4140

Liu, C., Zhang, L., Liu, H., and Cheng, K. (2017). Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. *J. Control. Rel.* 266, 17–26. doi: 10.1016/j.jconrel.2017.09.012

Liu, L., Yi, H., He, H., Pan, H., Cai, L., and Ma, Y. (2017). Tumor associated macrophage-targeted microRNA delivery with dual-responsive polypeptide nanovectors for anti-cancer therapy. *Biomaterials* 134, 166–179. doi: 10.1016/j.biomaterials.2017.04.043

Look, M., Saltzman, W. M., Craft, J., and Fahmy, T. M. (2014). The nanomaterial-dependent modulation of dendritic cells and its potential influence on therapeutic immunosuppression in lupus. *Biomaterials* 35, 1089–1095. doi: 10.1016/j.biomaterials.2013.10.046

Lu, C., Han, H. D., Mangala, L. S., Ali-Fehmi, R., Newton, C. S., Ozbun, L., et al. (2010). Regulation of tumor angiogenesis by EZH2. *Cancer Cell* 18, 185–197. doi: 10.1016/j.ccr.2010.06.016

Lu, C., Stewart, D. J., Lee, J. J., Ji, L., Ramesh, R., Jayachandran, G., et al. (2012). Phase I clinical trial of systemically administered TUSC2 (FUS1)-nanoparticles mediating functional gene transfer in humans. *PLoS One* 7:e34833. doi: 10.1371/journal.pone.0034833

Mahajan, V., Gaymalov, Z., Alakhova, D., Gupta, R., Zucker, I. H., and Kabanov, A. V. (2016). Horizontal gene transfer from macrophages to ischemic muscles upon delivery of naked DNA with pluronic block copolymers. *Biomaterials* 75, 58–70. doi: 10.1016/j.biomaterials.2015.10.002

Martinez, F. O., Helming, L., and Gordon, S. (2009). Alternative activation of macrophages: an immunologic functional perspective. *Annu. Rev. Immunol.* 27, 451–483. doi: 10.1146/annurev.immunol.021908.132532

McKenzie, E. J., Taylor, P. R., Stillion, R. J., Lucas, A. D., Harris, J., Gordon, S., et al. (2007). Mannose receptor expression and function define a new population of murine dendritic cells. *J. Immunol.* 178, 4975–4983. doi: 10.4049/jimmunol.178.8.4975

Miao, L., Li, J., Liu, Q., Feng, R., Das, M., Lin, C. M., et al. (2017a). Transient and local expression of chemokine and immune checkpoint traps to treat pancreatic cancer. *ACS Nano* 11, 8690–8706. doi: 10.1021/acsnano.7b01786

Miao, L., Liu, Q., Lin, C. M., Luo, C., Wang, Y., Liu, L., et al. (2017b). Targeting tumor-associated fibroblasts for therapeutic delivery in desmoplastic tumors. *Cancer Res.* 77, 719–731. doi: 10.1158/0008-5472.CAN-16-0866

Miao, L., Newby, J. M., Lin, C. M., Zhang, L., Xu, F., Kim, W. Y., et al. (2016). The binding site barrier elicited by tumor-associated fibroblasts interferes disposition of nanoparticles in stroma-vessel type tumors. *ACS Nano* 10, 9243–9258. doi: 10.1021/acsnano.6b02776

Miao, L., Wang, Y., Lin, C. M., Xiong, Y., Chen, N., Zhang, L., et al. (2015). Nanoparticle modulation of the tumor microenvironment enhances therapeutic efficacy of cisplatin. *J. Control. Release* 217, 27–41. doi: 10.1016/j.jconrel.2015.08.027

Miller, M. A., Zheng, Y. R., Gadde, S., Pfirschke, C., Zope, H., Engblom, C., et al. (2015). Tumour-associated macrophages act as a slow-release reservoir of nano-therapeutic Pt(IV) pro-drug. *Nat. Commun.* 6:8692. doi: 10.1038/ncomms9692

Mizrahy, S., Hazan-Halevy, I., Landesman-Milo, D., Ng, B. D., and Peer, D. (2017). Advanced strategies in immune modulation of cancer using lipid-based nanoparticles. *Front. Immunol.* 8:69. doi: 10.3389/fimmu.2017.00069

Moffett, H. F., Coon, M. E., Radtke, S., Stephan, S. B., McKnight, L., Lambert, A., et al. (2017). Hit-and-run programming of therapeutic cytoreagents using mRNA nanocarriers. *Nat. Commun.* 8:389. doi: 10.1038/s41467-017-00505-8

Morse, M. A., Chapman, R., Powderly, J., Blackwell, K. L., Keler, T., Green, J., et al. (2011). Phase I study utilizing a novel antigen-presenting cell-targeted vaccine with Toll-like receptor stimulation to induce immunity to self antigens in cancer patients. *Clin. Cancer Res.* 17, 4844–4853. doi: 10.1158/1078-0432.CCR-11-0891

Movahedi, K., Laoui, D., Gysemans, C., Baeten, M., Stangé, G., Van den Bossche, J., et al. (2010). Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C (high) monocytes. *Cancer Res.* 70, 5728–5739. doi: 10.1158/0008-5472.CAN-09-4672

Movahedi, K., Schoonooghe, S., Laoui, D., Houbracken, I., Waelput, W., Breckpot, K., et al. (2012). Nanobody-based targeting of the macrophage mannose receptor for effective in vivo imaging of tumor-associated macrophages. *Cancer Res.* 72, 4165–4177. doi: 10.1158/0008-5472.CAN-11-2994

Murphy, E. A., Majeti, B. K., Barnes, L. A., Makale, M., Weis, S. M., Lutu-Fuga, K., et al. (2008). Nanoparticle-mediated drug delivery to tumor vasculature suppresses metastasis. *Proc. Natl. Acad. Sci. U.S.A.* 105, 9343–9348. doi: 10.1073/pnas.0803728105

Nishikawa, M., and Huang, L. (2001). Nonviral vectors in the new millennium: delivery barriers in gene transfer. *Hum. Gene Ther.* 12, 861–870. doi: 10.1089/104303401750195836

Noy, R., and Pollard, J. W. (2014). Tumor-associated macrophages: from mechanisms to therapy. *Immunity* 41, 49–61. doi: 10.1016/j.immuni.2014.06.010

Ortega, R. A., Barham, W. J., Kumar, B., Tikhomirov, O., McFadden, I. D., Yull, F. E., et al. (2015). Biocompatible mannosylated endosomal-escape nanoparticles enhance selective delivery of short nucleotide sequences to tumor associated macrophages. *Nanoscale* 7, 500–510. doi: 10.1039/c4nr03962a

Ortega, R. A., Barham, W., Sharman, K., Tikhomirov, O., Giorgio, T. D., and Yull, F. E. (2016). Manipulating the NF-κB pathway in macrophages using mannosylated, siRNA-delivering nanoparticles can induce immunostimulatory and tumor cytotoxic functions. *Int. J. Nanomedicine* 11, 2163–2177. doi: 10.2147/IJN.S93483

Pecot, C. V., Calin, G. A., Coleman, R. L., Lopez-Berestein, G., and Sood, A. K. (2011). RNA interference in the clinic: challenges and future directions. *Nat. Rev. Cancer* 11, 59–67. doi: 10.1038/nrc2966

Pecot, C. V., Rupaimoole, R., Yang, D., Akbani, R., Ivan, C., Lu, C., et al. (2013). Tumour angiogenesis regulation by the miR-200 family. *Nat. Commun.* 4:2427. doi: 10.1038/ncomms3427

Peer, D., Park, E. J., Morishita, Y., Carman, C. V., and Shimaoka, M. (2008). Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. *Science* 319, 627–630. doi: 10.1126/science.1149859

Prabhakar, U., Maeda, H., Jain, R. K., Sevick-Muraca, E. M., Zamboni, W., Farokhzad, O. C., et al. (2013). Challenges and key considerations of the enhanced permeability and retention effect for nanomedicine drug delivery in oncology. *Cancer Res.* 73, 2412–2417. doi: 10.1158/0008-5472.can-12-4561

Qian, B.-Z., Li, J., Zhang, H., Kitamura, T., Zhang, J., Campion, L. R., et al. (2011). CCL2 recruits inflammatory monocytes to facilitate breast tumor metastasis. *Nature* 475, 222–225. doi: 10.1038/nature10138

Qian, Y., Qiao, S., Dai, Y., Xu, G., Dai, B., Lu, L., et al. (2017). Molecular-targeted immunotherapeutic strategy for melanoma via dual-targeting nanoparticles delivering small interfering RNA to tumor-associated macrophages. *ACS Nano* 11, 9536–9549. doi: 10.1021/acsnano.7b05465

Raghuwanshi, D., Mishra, V., Das, D., Kaur, K., and Suresh, M. R. (2012). Dendritic cell targeted chitosan nanoparticles for nasal DNA immunization against SARS CoV nucleocapsid protein. *Mol. Pharm.* 9, 946–956. doi: 10.1021/mp200553x

Ramishetti, S., Kedmi, R., Goldsmith, M., Leonard, F., Sprague, A. G., Godin, B., et al. (2015). Systemic gene silencing in primary T lymphocytes using targeted lipid nanoparticles. *ACS Nano* 9, 6706–6716. doi: 10.1021/acsnano.5b02796

Rice, J., Ottensmeier, C. H., and Stevenson, F. K. (2008). DNA vaccines: precision tools for activating effective immunity against cancer. *Nat. Rev. Cancer* 8, 108–120. doi: 10.1038/nrc2326

Rietwyk, S., and Peer, D. (2017). Next-generation lipids in RNA interference therapeutics. *ACS Nano* 11, 7572–7586. doi: 10.1021/acsnano.7b04734

Rupaimoole, R., and Slack, F. J. (2017). MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat. Rev. Drug Discov.* 16, 203–222. doi: 10.1038/nrd.2016.246

Sánchez-Rivera, F. J., and Jacks, T. (2015). Applications of the CRISPR-Cas9 system in cancer biology. *Nat. Rev. Cancer* 15, 387–395. doi: 10.1038/nrc3950

Santel, A., Aleku, M., Keil, O., Endruschat, J., Esche, V., Durieux, B., et al. (2006). RNA interference in the mouse vascular endothelium by systemic administration of siRNA-lipoplexes for cancer therapy. *Gene Ther.* 13, 1360–1370. doi: 10.1038/sj.gt.3302778

Sehgal, K., Dhodapkar, K. M., and Dhodapkar, M. V. (2014a). Targeting human dendritic cells in situ to improve vaccines. *Immunol. Lett.* 162, 59–67. doi: 10.1016/j.imlet.2014.07.004

Sehgal, K., Ragheb, R., Fahmy, T. M., Dhodapkar, M. V., and Dhodapkar, K. M. (2014b). Nanoparticle-mediated combinatorial targeting of multiple human dendritic cell (DC) subsets leads to enhanced T Cell activation via IL-15–dependent DC crosstalk. *J. Immunol.* 193, 2297–2305. doi: 10.4049/jimmunol.1400489

Shann, S. Y., Lau, C. M., Barham, W. J., Onishko, H. M., Nelson, C. E., Li, H., et al. (2013). Macrophage-specific RNAi targeting via 'click', mannosylated polymeric micelles. *Mol. Pharm.* 10, 975–987. doi: 10.1021/mp300434e

Sica, A., Schioppa, T., Mantovani, A., and Allavena, P. (2006). Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur. J. Cancer* 42, 717–727. doi: 10.1016/j.ejca.2006.01.003

Sidi, A. A., Ohana, P., Benjamin, S., Shalev, M., Ransom, J. H., Lamm, D., et al. (2008). Phase I/II marker lesion study of intravesical BC-819 DNA plasmid in H19 over expressing superficial bladder cancer refractory to bacillus Calmette-Guerin. *J. Urol.* 180, 2379–2383. doi: 10.1016/j.juro.2008.08.006

Smaldone, M. C., and Davies, B. J. (2010). BC-819, a plasmid comprising the H19 gene regulatory sequences and diphtheria toxin A, for the potential targeted therapy of cancers. *Curr. Opin. Mol. Ther.* 12, 607–616.

Stahl, P. D., and Ezekowitz, R. A. B. (1998). The mannose receptor is a pattern recognition receptor involved in host defense. *Curr. Opin. Immunol.* 10, 50–55. doi: 10.1016/S0952-7915(98)80031-9

Stewart, P. L., and Nemerow, G. R. (2007). Cell integrins: commonly used receptors for diverse viral pathogens. *Trends Microbiol.* 15, 500–507. doi: 10.1016/j.tim.2007.10.001

Storm, G., Belliot, S. O., Daemen, T., and Lasic, D. D. (1995). Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system. *Adv. Drug Deliv. Rev.* 17, 31–48. doi: 10.1002/asia.201800149

Ueno, N. T., Bartholomeusz, C., Xia, W., Anklesaria, P., Bruckheimer, E. M., Mebel, E., et al. (2002). Systemic gene therapy in human xenograft tumor models by liposomal delivery of the E1A gene. *Cancer Res.* 62, 6712–6716.

van Zandwijk, N., Pavlakis, N., Kao, S. C., Linton, A., Boyer, M. J., Clarke, S., et al. (2017). Safety and activity of microRNA-loaded minicells in patients

with recurrent malignant pleural mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study. *Lancet Oncol.* 18, 1386–1396. doi: 10.1016/S1470-2045(17)30621-6

Walkey, C. D., Olsen, J. B., Guo, H., Emili, A., and Chan, W. C. (2012). Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. *J. Am. Chem. Soc.* 134, 2139–2147. doi: 10.1021/ja2084338

Walters, A. A., Somavarapu, S., Riitho, V., Stewart, G. R., Charleston, B., Steinbach, F., et al. (2015). Assessment of the enhancement of PLGA nanoparticle uptake by dendritic cells through the addition of natural receptor ligands and monoclonal antibody. *Vaccine* 33, 6588–6595. doi: 10.1016/j.vaccine.2015.10.093

Wang, C., Li, P., Liu, L., Pan, H., Li, H., Cai, L., et al. (2016). Self-adjuvanted nanovaccine for cancer immunotherapy: role of lysosomal rupture-induced ROS in MHC class I antigen presentation. *Biomaterials* 79, 88–100. doi: 10.1016/j.biomaterials.2015.11.040

Wang, J. J., Zeng, Z. W., Xiao, R. Z., Xie, T., Zhou, G. L., Zhan, X. R., et al. (2011). Recent advances of chitosan nanoparticles as drug carriers. *Int. J. Nanomed.* 6, 765–774. doi: 10.2147/IJN.S17296

Wayteck, L., Xiong, R., Braeckmans, K., De Smedt, S. C., and Raemdonck, K. (2017). Comparing photoporation and nucleofection for delivery of small interfering RNA to cytotoxic T cells. *J. Control. Release* 267, 154–162. doi: 10.1016/j.jconrel.2017.08.002

Weissleder, R., Nahrendorf, M., and Pittet, M. J. (2014). Imaging macrophages with nanoparticles. *Nat. Mater.* 13, 125–138. doi: 10.1038/nmat3780

Wittrup, A., and Lieberman, J. (2015). Knocking down disease: a progress report on siRNA therapeutics. *Nat. Rev. Genet.* 16, 543–552. doi: 10.1038/nrg3978

Working, P., Newman, M., Huang, S., Mayhew, E., Vaage, J., and Lasic, D. (1994). Pharmacokinetics, biodistribution and therapeutic efficacy of doxorubicin encapsulated in Stealth® liposomes (Doxil®). *J. Liposome Res.* 4, 667–687. doi: 10.3109/08982109409037065

Xiao, C., and Rajewsky, K. (2009). MicroRNA control in the immune system: basic principles. *Cell* 136, 26–36. doi: 10.1016/j.cell.2008.12.027

Xie, J., Xu, C., Kohler, N., Hou, Y., and Sun, S. (2007). Controlled PEGylation of monodisperse Fe3O4 nanoparticles for reduced non-specific uptake by macrophage cells. *Adv. Mater.* 19, 3163–3166. doi: 10.1002/adma.200701975

Yamamoto, A., Kormann, M., Rosenecker, J., and Rudolph, C. (2009). Current prospects for mRNA gene delivery. *Eur. J. Pharm. Biopharm.* 71, 484–489. doi: 10.1016/j.ejpb.2008.09.016

Yin, H., Kanasty, R. L., Eltoukhy, A. A., Vegas, A. J., Dorkin, J. R., and Anderson, D. G. (2014). Non-viral vectors for gene-based therapy. *Nat. Rev. Genet.* 15, 541–555. doi: 10.1038/nrg3763

Yoo, G. H., Hung, M.-C., Lopez-Berestein, G., LaFollette, S., Ensley, J. F., Carey, M., et al. (2001). Phase I trial of intratumoral liposome E1A gene therapy in patients with recurrent breast and head and neck cancer. *Clin. Cancer Res.* 7, 1237–1245.

Zanta, M. A., Belguise-Valladier, P., and Behr, J.-P. (1999). Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 96, 91–96. doi: 10.1073/pnas.96.1.91

Zhang, M., Gao, Y., Caja, K., Zhao, B., and Kim, J. A. (2015a). Non-viral nanoparticle delivers small interfering RNA to macrophages in vitro and in vivo. *PLoS One* 10:e0118472. doi: 10.1371/journal.pone.0118472

Zhang, M., Yan, L., and Kim, J. (2015b). Modulating mammary tumor growth, metastasis and immunosuppression by siRNA-induced MIF reduction in tumor microenvironment. *Cancer Gene Ther.* 22, 463–475. doi: 10.1038/cgt.2015.42

Zuckerman, J. E., and Davis, M. E. (2015). Clinical experiences with systemically administered siRNA-based therapeutics in cancer. *Nat. Rev. Drug Discov.* 14, 843–857. doi: 10.1038/nrd4685

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The Potential of Nanotechnology in Medically Assisted Reproduction

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Reproductive medicine is a field of science which searches for new alternatives not only to help couples achieve pregnancy and preserve fertility, but also to diagnose and treat diseases which can impair the normal operation of the reproductive tract. Assisted reproductive technology (ART) is a set of methodologies applied to cases related to infertility. Despite being highly practiced worldwide, ART presents some challenges, which still require special attention. Nanotechnology, as a tool for reproductive medicine, has been considered to help overcome some of those impairments. Over recent years, nanotechnology approaches applied to reproductive medicine have provided strategies to improve diagnosis and increase specificity and sensitivity. For *in vitro* embryo production, studies in non-human models have been used to deliver molecules to gametes and embryos. The exploration of nanotechnology for ART would bring great advances. In this way, experiments in non-human models to test the development and safety of new protocols using nanomaterials are very important for informing potential future employment in humans. This paper presents recent developments in nanotechnology regarding impairments still faced by ART: ovary stimulation, multiple pregnancy, and genetic disorders. New perspectives for further use of nanotechnology in reproductive medicine studies are also discussed.

Keywords: assisted reproductive technologies, nanotechnology, nanobiotechnology, multiple pregnancy, *in vitro* maturation, gene therapy, embryology

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REPRODUCTIVE MEDICINE AND NANOTECHNOLOGY

Infertility and subfertility defined as the difficulty to conceive are conditions affecting people worldwide. The World Health Organization considers infertile couples those who fail to achieve a clinical pregnancy after, at least, 1 year of regular unprotected sexual intercourse (Zegers-Hochschild et al., 2017). Couples who experience these difficulties can turn to reproductive medicine technologies to help solve the problem. One of the most revolutionary treatments in this area is assisted reproductive technology (ART) comprising of *in vitro* embryo production (IVEP).

Regarding reproductive medicine, nanotechnology can be very useful in the development of non-invasive detection, diagnosis, and minimally invasive treatment of infertility-related disorders (oncological or non-oncological) (Barkalina et al., 2014a). To improve diagnostics, nanotechnology is applied mainly to the development and improvement of nanobiosensors and imaging techniques. Nanobiosensors are devices capable of identifying antigens, proteins, nucleic acids, and reactive oxygen and nitrogen species with quickness and sensitivity (Shi et al., 2007; Zhu et al., 2015).

These technologies are underlying the development of interesting 'lab-on-a-chip' tools. Besides advantages of nanotechnology, this tool requires small volumes of analyte and reagents (Craighead, 2006; Hill and Li, 2017). The functionalization of zinc oxide nanoroads – gold nanoparticles (Gasparotto et al., 2017), iron oxide nanoparticles (Pal et al., 2015), and silica-coated gold nanoparticles with cadmium selenide quantum dots (Johari-Ahar et al., 2015) with anti-CA125 antibodies represent successful strategies to develop higher sensitivity tools for ovarian cancer detection. In addition, anti-HE4 antibody attached to silver nanoparticles was also used to develop a fast, specific, and stable ovarian cancer detection system (Yuan et al., 2012).

Biosensors using anti-PSA antibodies to detect PSA antigen represent one of the most used strategies for detection of prostate cancer. Gold nanoparticles functionalized with anti-PSA antibodies have been used in a bio-barcode assay showing ultrasensitivity (Thaxton et al., 2009) and in silicon nanowire field-effect transistors providing real-time prostate cancer detection (Presnova et al., 2017). Gold nanoparticles and anti-PSA antibodies supported in graphene oxide (Pal and Khan, 2017) or bound in cuprous oxide@ceric dioxide core–shell nanocomposites (Li et al., 2017) were also used to develop novel, accurate, and sensitive electrochemical immunosensors.

Diagnostic imaging has been improved by metallic and nanostructured particles, as these nanomaterials have great benefits compared to contrast agents. Iron oxide is one of the main contrast agents used for magnetic resonance imaging (MRI), and when nanostructured, it can be functionalized for additional benefits. Iron oxide nanoparticles can be formed in poly(vinyl alcohol), rendering them degradable over time (Bannerman et al., 2017) or, as showed in tumor xenograft animal models, can be associated to diatoms to improve tumor retention when a magnetic field is applied (Todd et al., 2014). In addition, iron oxide nanoparticles can be directed to a tumor site. For example, the functionalization of superparamagnetic iron oxide nanoparticles (SPIONs) with anti-prostate-specific membrane antigen (PSMA) increased the detection limit and the sensitivity of MRI in prostate tumor cell culture (Sillerud, 2016). In addition to iron oxide, other nanomaterials already tested in cell culture and/or animal models can be used as contrast agents to enhance imaging diagnostics, including gold nanoparticles (Indrasekara et al., 2013; Cole et al., 2015), carbon nanotubes (Liu et al., 2007; Vittorio et al., 2011), liposomes (Martina et al., 2005; Mukundan et al., 2006), dendrimers (Miyake et al., 2015), and quantum dots (Guo et al., 2014; Yao et al., 2016).

In the treatment of oncological diseases of the reproductive system, recent drug delivery and cell-target strategies have been developed. For example, one of the main anticancer drugs used, doxorubicin, has been associated to nanoformulations to increase its efficacy. These include mesoporous silica nanoparticles (Guo et al., 2017) and lipid-coated mesoporous iron oxide–based magnetic nanoassemblies (Pradhan et al., 2016) tested in human cell culture and xenograft mouse models, respectively. PEGylated liposomes have also been tested for cervical and ovarian cancer using human cells (Sriraman et al., 2016). Magnetic nanoparticles (Hua et al., 2017) have been used to treat cervical cancer in human cell cultures and xenograft mice. Other strategies include the delivering of siRNA in cationic dendritic starch (Engelberth et al., 2017), layer-by-layer engineering of upconversion nanoparticles (Lin et al., 2017), and mesoporous silica nanoparticles (Roberts et al., 2017) resulting in improved cell death in human ovarian cancer cells.

For non-oncological diseases of the reproductive system, some alternatives were tested in human cell culture. To treat uterine leiomyoma, strategies included the use of magnetic nanoparticles complexed to adenovirus (Shalaby et al., 2016) and nanoparticles loaded with 2-methoxyestradiol (Ali et al., 2013). In animal models, carbosilane dendrimer (Chonco et al., 2012) and nanoparticles-in-film (Cunha-Reis et al., 2016) were tested for the treatment of HIV infections. Another condition that could impair fertility is endometriosis, and the strategies already generated using nanomaterials are listed in **Table 1**.

In the field of fertility preservation, nanotechnology was shown to improve the potential of cryopreserved human immature testicular tissue to restore fertility. Dextran–chitosan nanoparticles loaded with vascular endothelial growth factor (VEGF) were tested for tissue engraftment after cryopreservation of the tissue in mice, resulting in higher vascular density and spermatogonia recovery in transplanted tissues (Poels et al., 2016). For female gametes, an interesting strategy for swine oocyte cryopreservation was developed. The addition of low concentrations of hydroxy apatite nanoparticles (less than 0.5%) in cryoprotectant agents increased the developmental rate of vitrified/devitrified germinal vesicles oocytes (Li et al., 2016). These are a few of the different contributions that nanotechnology has been giving to medically assisted reproduction.

POTENTIAL CONTRIBUTIONS OF NANOTECHNOLOGY TO ASSISTED REPRODUCTIVE TECHNOLOGY

Although ART is successfully applied as a clinical treatment worldwide, some challenges remain. Because of this, strategies developed in animal models are highly important for identifying new alternatives to overcome these problems. When it comes to ART, embryo development in mammalian models is highly similar to humans (Niemann and Wrenzycki, 2000; Barkalina et al., 2016). Lagomorph, murine, swine, bovine, and non-human primates are the main species used to study IVEP techniques to be applied to humans.

Similarly, the implementation of nanotechnology, which has already been developed for non-human animals, could be applied to assisted reproduction in humans (Langbeen et al., 2015; Barkalina et al., 2016). As mentioned previously, this technology has already been tested and used in sectors adjacent to reproductive medicine. Therefore, the main

TABLE 1 | Strategies for treating endometriosis using materials in nanoscale.

Nanomaterial	Strategy	Animal model (cell type)	Main results	Bibliographic reference
Poly(lactic-co-glycolic acid) (PLGA) nanoparticles	PLGA nanoparticle to carry an anti-CD4 antibody	Female C57 endometriosis mouse model	The proposed treatment inhibited IL-10 and TGF-beta secreted by CD4+CD25+Treg cells.	Liu et al., 2017
Polyethylenimine-grafted chitosan oligosaccharide (CSO-PEI) with hyaluronic acid (HA)	Gene delivery of aquaporin 2 – small interfering RNA by polymeric nanoparticles	Ishikawa (IK) cells and female Sprague-Dawley rats with induced endometrial lesions	The tested strategy decreased the endometriotic lesion sizes with atrophy and degeneration of the ectopic endometrium. Also, the epithelial cells of ectopic endometrium showed a significant decrease of CD44 expression.	Zhao et al., 2016
Polyvinylpyrrolidone (PVP K-30)	Nanoencapsulation of copaiba oil-resin	Primary cell cultures of endometrial stromal cells (ESCs) obtained from ectopic endometrium of patients with endometriosis (EuESCs), ESCs obtained from ectopic endometrium of patients without endometriosis (CESCs) and ESCs from endometriotic lesions (EctESCs)	The proposed method reduced viability and proliferation of endometriotic cell cultures upon COPA nanocomposite treatment.	De Almeida Borges et al., 2016
Poly(lactic-co-glycolic) (PLGA) nanoparticles	Nanoencapsulation of epigallocatechin gallate and doxycycline	Human skin keratinocyte (HACAT) cell line and Swiss albino female mice	The proposed treatment decreased oxidative stress, matrix metalloproteinase activity, angiogenesis, endometrial gland presence and microvessel density, and improved oocyte quality.	Singh et al., 2015
Unmodified silica nanoparticles (UMNPs) and modified by aminopropyl groups silica nanoparticles (AMNPs)	Nanoencapsulation of glucosaminyl muramyl dipeptide (N-acetylglucosaminyl-N-acetylmuramyl-L-alanyl-D-isoglutamine) (GMDP)	Peritoneal mononuclear cells (MNC) derived from peritoneal fluid of women with endometriosis	The proposed strategy improved immunomodulatory effect of GMDP by the nanoencapsulation in silica nanoparticles.	Antsiferova et al., 2013
Cerium oxide nanoparticles (nanoceria)	Mitigation of endometrial lesions by nanoceria	CD-1 strain Swiss Albino female mice endometriosis induced	The nanoceria decreased oxidative stress, inhibited angiogenesis, and protected oocytes from endometriosis-related adverse effects.	Chaudhury et al., 2013
Chitosan-derived polymeric micelles with glycolipid-like structure	Gene delivery of pigment epithelium derived factor gene by micelles	Female Sprague-Dawley rats with induced endometrial lesions	The proposed gene therapy caused a decrease in the sizes of the endometriotic lesions, an atrophy and degeneration of ectopic endometrium, a significantly decrease in microvessel density and increased index of apoptotic in endometriotic lesions.	Zhao et al., 2012

challenges of ART nowadays are how nanotechnology can intervene in order to boost the techniques already used today.

OVARIAN STIMULATION AND *IN VITRO* MATURATION

To perform ART procedures, ovarian stimulation is routinelly required in order to obtain a higher number of oocytes and increase the chances of embryo production to enable the selection of the best quality embryos for transfer (Fauser et al., 2005). Despite the increased number of oocytes that can be obtained using this procedure, some impairment has been observed. In addition to the high costs and the modest success rates, there are also potential health risks for the patients such as ovarian hyperstimulation syndrome in case of hyperresponse to ovarian stimulation (Huang et al., 2010).

In vitro maturation (IVM) is one of the most promising strategies for overcoming problems related to ovarian stimulation. Oocyte maturation consists of modification of

genomic structures, organelle restructurations, and molecular production to allow the gamete to receive spermatozoa for fertilization (Fulka et al., 1998; Mao et al., 2014). Using the IVM technique, immature oocytes are collected from ovaries of non-stimulated patients, followed by selection and exposure to IVM medium consisting of a base medium for cell culture supplemented with hormones, including FSH, LH, and estradiol. However, despite its clinical utility and successfull application in farm animals (Goto et al., 1988; Hwu et al., 1998), IVM of human oocytes remains an experimental approach not widely accepted in fertility clinics worldwide (Chang et al., 2014; Tannus et al., 2017). This is likely due to the lower pregnancy and live birth rates using *in vitro* compared to *in vivo* maturated oocytes, likely due to inadequacies of the culture media (Combelles et al., 2002; Ortega-Hrepich et al., 2013).

It is well established that embryo quality is dependent on oocyte quality (Lonergan et al., 2003; Ferris et al., 2016). In addition, correct and complete oocyte maturation is essential to efficient embryo production. Regarding IVM, the process can be disrupted by excess production of ROS, which is one of the major causes of oocyte depletion (Tamura et al., 2008; Karuputhula et al., 2013). For IVM, the addition of antioxidants is helpful, but these molecules may not exert their function with high efficiency due to their instability in *in vitro* enviroment, making utilization of nanomaterials an interesting strategy for molecule protection (Lucas et al., 2015; Komninou et al., 2016; Remião et al., 2016; Duarah et al., 2017; Manconi et al., 2017). One study from our group has show increased cleavage and blastocyst production rates, decreased ROS levels, and decreased the number of apoptotic cells/blastocyst when bovine oocytes were supplemented with nanoencapsulated melatonin in a IVM medium (Remião et al., 2016).

In another study, tretinoin was nanoencapsulated in lipid-core nanocapsules (LNC) and supplementation with the minor tested concentration (0.25 μM) in IVM medium was benneficial for bovine oocytes, resulting in higher cleavage and blastocyst rates, decreased P66Shc protein levels (the 66-kDa isoform of the growth-factor adapter Shc), and decreased ROS production. These benefits were not observed using the same concentration of non-encapsulated tretinoin (Lucas et al., 2015). Therefore, this represents a potential strategy for increasing the effectivness of human IVM and IVEP.

MULTIPLE PREGNANCY

Multiple pregnancies are a current problem in ART. The incidence of multiple pregnancies is related to pre-term birth, birth of babies with low weight and other complications, and risks to mothers and babies (Fauser et al., 2005; Vulliemoz et al., 2012). The high incidence of multiple pregnancies when using ART is related to the fact that sometimes more than one embryo is transferred into the female reproductive tract (Friedman et al., 2011; Mersereau et al., 2017).

In order to overcome the multiple pregnancy problems in ART, one alternative is the transfer of single embryos performed at a higher frequency (Mancuso et al., 2016). The methodologies assisting this condition are IVM, *in vitro* blastocyst culture, and embryo cryopreservation, techniques that have been highly studied in small and large animals and have been utilized commercially for many years (Sinclair, 2008).

Preimplantational genetic screening (PGS) and preimplantational genetic diagnosis (PGD) can also be useful to avoid multiple pregnancies, by discarding embryos with genetic disorders. To perform PGS and PGD, embryos are biopsied and evaluated using techniques such as karyotyping, fluorescent *in situ* hybridization (FISH), quantitative polymerase chain reaction (qPCR), array comparative genomic hybridization (aCGH), and next generation sequencing (NGS) (Chen et al., 2017).

Nanotechnology can help researches improve the application of PGS and PGD. Although highly employed, the current detection methods could be more sensitive and specific, more affordable and accessible to patients, faster, and easier to use to facilitate use in human reproduction clinics. Gold, silver, carbon, and magnetic nanomaterials are the main materials used to develop new methods of genetic diagnostics (Zhu et al., 2015). Nanotechnology combined with colorimetric (Stoeva et al., 2006) and electrochemical (Ozsoz et al., 2003) methods for nucleic acid analysis and detection has brought more sensitivity, lower cost, and increased simplicity and portability to diagnostics. This and other strategies recently developed for DNA analysis can be applied in the future to simplify PGD and PGS diagnostic procedures.

Another strategy for embryo selection is the culture of human embryos until day 5/6, when they reach the blastocyst stage. It has been previously shown that blastocyst transfer (day 5/6) presents better results than cleavage embryos (day 2/3) (Abuzeid et al., 2014; Yin et al., 2017). However, some clinics transfer embryos at the cleavage stage because most embryos fail to reach day 5/6 due to difficulties in mimicking the complexities of the *in vivo* environment (Alper et al., 2001; Tsirigotis, 1998). *In vitro* culture and manipulation of gametes and embryos stimulates production of exogenous ROS and leads to oxidative stress, reducing embryo quality (Agarwal et al., 2006; Truong et al., 2016). To overcome the challenge of embryo culture leading up to the blastocyst stage, research groups have looked for alternative approaches to improve *in vitro* embryo culture, including the addition of antioxidant molecules to the medium.

Studies on IVEP in animal models indicate antioxidant supplementation in medium is beneficial for blastocyst production. Antioxidants presenting beneficial effects in animal model *in vitro* embryo cultures include L-carnitine (Abdelrazik et al., 2009), hyaluronan (Romek et al., 2017), resveratrol (Salzano et al., 2014), and melatonin (Wang et al., 2013, 2014). However, in the case of bovine IVM, nanotechnology provides interesting alternatives for protecting of these molecules in *in vitro* environments (Lucas et al., 2015; Komninou et al., 2016; Remião et al., 2016). A recent publication confirmed this approach may represent a relevant alternative: supplementation of IVC medium with melatonin-loaded LNC increased embryo

quality and blastocyst hatching in a bovine model (Komninou et al., 2016). This strategy is beneficial since the nanocapsules are biodegradable and do not result in toxicity when exposed to bovine oocytes (Lucas et al., 2017) or administered intradermally in rats (Bulcão et al., 2014).

GENETIC DISORDERS

The development and improvement of genome editing technology in the last few years has introduced gene therapy as a pre-emptive solution for correction of genetic anomalies. Monogenic diseases may be easily corrected using gene therapy, as they are caused by a single defective gene (Ma et al., 2017). Some monogenic diseases have already been targeted by gene therapy techniques, including lipoprotein lipase deficiency (Gaudet et al., 2016), hemophilia B (Nathwani et al., 2017), β-hemoglobinopathies (Negre et al., 2016), Wiskott–Aldrich syndrome (Aiuti et al., 2013; Morris et al., 2017), and inherited retinal degenerations (Gupta and Huckefeldt, 2017), although these diseases have not been treated in embryos.

Two recent reports have already shown the possibility of gene editing human embryos to correct genetic disorders. The studies used the CRISPR-Cas9 method to fix the human β-globin gene (Liang et al., 2015) and heterozygous *MYBPC3* mutation (Ma et al., 2017), mutations responsible for β-thalassemia and hypertrophic cardiomyopathy, respectively. Although these studies have raised ethical concerns and the technology is still experimental, without proven efficacy and safety, both publications bring an important alternative to reproductive medicine through the treatment of diseases that until now were considered incurable (Ishii, 2017).

Nanotechnology development has resulted in some interesting non-viral strategies for molecule delivery in cells (Barkalina et al., 2015) contributing to the optimization of gene editing. One of them is the study of Sun et al. (2015) that delivered the Cas9 protein and a guide RNA through a DNA nanoclew to human osteosarcoma tumors in mice. Diverse studies have shown efficient gene delivery in mammalian cells via nanomaterials (Guan and Rosenecker, 2017; Riley and Vermerris, 2017). To produce genetically modified embryos, nanomaterials can be used to increase the efficiency of gene transfer via sperm mediated gene transfer. Silica nanoparticles (Barkalina et al., 2014b), magnetic iron nanoparticles (Kim et al., 2010), halloysite clay nanotubes (Campos et al., 2011), and poly(vinyl alcohol)-coated iron oxide nanoparticles (Makhluf et al., 2008) have already shown promise for delivery of nucleic acids and/or proteins to bovine spermatozoa.

Single-cell embryos can also be directly modified using nanomaterials. Das et al. (2016) hypothesized that if single-cell stage zona-free bubaline embryos are transfected with commercial transfecting agents and developed until the blastocyst stage (Selokar et al., 2015), nanomaterials could also be used to introduce genes into embryos at this stage and condition. This could be an alternative to not only viral vectors, but also other expensive methods such as pronuclear microinjection (Das et al., 2016). However, more studies are needed before introducing this technology into practice due to the possible toxic effects.

CHALLENGES FOR THE USE OF NANOTECHNOLOGY IN REPRODUCTIVE SCIENCE

Nanotechnology has already and can continue to provide advantages for reproductive medicine. Despite the great solutions it can offer, some challenges still faces the use of this technology in medicine. As it is an emerging science, few studies have been performed to validate all the possibilities for treatments or diagnostics. One of the main questions that still need to be addressed regarding the use of nanotechnology is the toxicity it could cause. Despite new diagnostic methodologies being closer to being applied commercially, the ways in which nanomaterials are administered to organisms, embryos, or gametes need to be further studied.

Some nanomaterials are toxic to organism, mainly when exposure occurs during pregnancy and embryo development. For example, when pregnant mice are exposed to titanium dioxide nanomaterials, these materials can cross the placental barrier and cause anatomical defects in the fetuses (Melnik et al., 2013; Naserzadeh et al., 2017). In addition, silver nanoparticles decrease oestrogen plasma levels, increasing the number of resorbed fetuses (Campagnolo et al., 2017) and affecting embryonic growth (Austin et al., 2016). Carbon nanotubes also decrease the number of live fetuses per dam (Fujitani et al., 2015), the number of blood vessels on placenta, and increase the number of abortions (Qi et al., 2014).

Because of this, the utilization of these nanomaterials for reproductive proposes should be done carefully. One alternative is to search for additional nanomaterials that do not present toxicity, with biodegradable structures as the LNC and the dextran/chitosan nanoparticles, representing the most promising nanostructures for use in health applications.

FINAL CONSIDERATIONS

Despite the recent advances in assisted reproductive technologies, some challenges remain, mainly related to pregnancy rates, multiple births, and genetic disorders. To overcome these problems, new alternatives must be identified. Nanotechnology represents a valuable tool that must be explored further to help researchers identify solutions for reproductive medicine. Nanomaterials can bring specificity, practice, and sensibility to next-generation diagnostic and treatment modalities.

It is expected that, as in other areas of medicine, the employment of nanotechnology could be helpful and beneficial to patients. In addition, researchers must be encouraged to develop more *in vitro* and *in vivo* tests using animal models to test safety

and efficiency of these new methodologies. In addition, human clinical reproductive trials may also help accelerate commercial availability of new alternatives for ART.

AUTHOR CONTRIBUTIONS

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REFERENCES

Abdelrazik, H., Sharma, R., Mahfouz, R., and Agarwal, A. (2009). L-Carnitine decreases DNA damage and improves the *in vitro* blastocyst development rate in mouse embryos. *Fertil. Steril.* 91, 589–596. doi: 10.1016/j.fertnstert.2007.11.067

Abuzeid, M. I., Bolonduro, O., La Chance, J., Abozaid, T., Urich, M., Ullah, K., et al. (2014). Cumulative live birth rate and assisted reproduction: impact of female age and transfer day. *Facts Views Vis. Obgyn* 6, 145–149.

Agarwal, A., Said, T. M., Bedaiwy, M. A., Banerjee, J., and Alvarez, J. G. (2006). Oxidative stress in an assisted reproductive techniques setting. *Fertil. Steril.* 86, 503–512. doi: 10.1016/j.fertnstert.2006.02.088

Aiuti, A., Biasco, L., Scaramuzza, S., Ferrua, F., Cicalese, M. P., Baricordi, C., et al. (2013). Lentivirus-based gene therapy of hematopoietic stem cells in Wiskott-Aldrich Syndrome. *Science* 341:1233151. doi: 10.1126/science.1233151

Ali, H., Kalashnikova, I., White, M. A., Sherman, M., and Rytting, E. (2013). Preparation, characterization, and transport of dexamethasone-loaded polymeric nanoparticles across a human placental *in vitro* model. *Int. J. Pharm.* 454, 149–157. doi: 10.1016/j.ijpharm.2013.07.010

Alper, M. M., Brinsden, P., Fischer, R., and Wikland, M. (2001). To blastocyst or not to blastocyst? That is the question. *Hum. Reprod.* 16, 617–619. doi: 10.1093/humrep/16.4.617

Antsiferova, Y., Sotnikova, N., and Parfenyuk, E. (2013). Different effects of the immunomodulatory drug GMDP immobilized onto aminopropyl modified and unmodified mesoporous silica nanoparticles upon peritoneal macrophages of women with endometriosis. *Biomed Res. Int.* 2013:924362. doi: 10.1155/2013/924362

Austin, C. A., Hinkley, G. K., Mishra, A. R., Zhang, Q., Umbreit, T. H., Betz, M. W., et al. (2016). Distribution and accumulation of 10 nm silver nanoparticles in maternal tissues and visceral yolk sac of pregnant mice, and a potential effect on embryo growth. *Nanotoxicology* 10, 654–661. doi: 10.3109/17435390.2015.1107143

Bannerman, A. D., Li, X., and Wan, W. (2017). A "degradable" poly(vinyl alcohol) iron oxide nanoparticle hydrogel. *Acta Biomater.* 58, 376–385. doi: 10.1016/j.actbio.2017.05.018

Barkalina, N., Charalambous, C., Jones, C., and Coward, K. (2014a). Nanotechnology in reproductive medicine: emerging applications of nanomaterials. *Nanomedicine* 10, 921–938. doi: 10.1016/j.nano.2014.01.001

Barkalina, N., Jones, C., and Coward, K. (2016). Nanomedicine and mammalian sperm: lessons from the porcine model. *Theriogenology* 85, 74–82. doi: 10.1016/j.theriogenology.2015.05.025

Barkalina, N., Jones, C., Kashir, J., Coote, S., Huang, X., Morrison, R., et al. (2014b). Effects of mesoporous silica nanoparticles upon the function of mammalian sperm *in vitro*. *Nanomedicine* 10, 859–870. doi: 10.1016/j.nano.2013.10.011

Barkalina, N., Jones, C., Wood, M. J. A., and Coward, K. (2015). Extracellular vesicle-mediated delivery of molecular compounds into gametes and embryos: learning from nature. *Hum. Reprod. Update* 21, 627–639. doi: 10.1093/humupd/dmv027

Bulcão, R. P., de Freitas, F. A., Dallegrave, E., Venturini, C. G., Baierle, M., Durgante, J., et al. (2014). In vivo toxicological evaluation of polymeric nanocapsules after intradermal administration. *Eur. J. Pharm. Biopharm.* 86, 167–177. doi: 10.1016/j.ejpb.2013.04.001

Campagnolo, L., Massimiani, M., Vecchione, L., Piccirilli, D., Toschi, N., Magrini, A., et al. (2017). Silver nanoparticles inhaled during pregnancy reach and affect the placenta and the foetus. *Nanotoxicology* 11, 687–698. doi: 10.1080/17435390.2017.1343875

Campos, V. F., de Leon, P. M. M., Komninou, E. R., Dellagostin, O. A., Deschamps, J. C., Seixas, F. K., et al. (2011). NanoSMGT: transgene transmission into bovine embryos using halloysite clay nanotubes or nanopolymer to improve transfection efficiency. *Theriogenology* 76, 1552–1560. doi: 10.1016/j.theriogenology.2011.06.027

Chang, E. M., Song, H. S., Lee, D. R., Lee, W. S., and Yoon, T. K. (2014). *in vitro* maturation of human oocytes: its role in infertility treatment and new possibilities. *Clin. Exp. Reprod. Med.* 41, 41–46. doi: 10.5653/cerm.2014.41.2.41

Chaudhury, K., Babu, K. N., Singh, A. K., Das, S., Kumar, A., and Seal, S. (2013). Mitigation of endometriosis using regenerative cerium oxide nanoparticles. *Nanomedicine* 9, 439–448. doi: 10.1016/j.nano.2012.08.001

Chen, H.-F., Chen, S.-U., Ma, G.-C., Hsieh, S.-T., Tsai, H.-D., Yang, Y.-S., et al. (2017). Preimplantation genetic diagnosis and screening: current status and future challenges. *J. Formos. Med. Assoc.* doi: 10.1016/j.jfma.2017.08.006 [Epub ahead of print].

Chonco, L., Pion, M., Vacas, E., Rasines, B., Maly, M., Serramía, M. J., et al. (2012). Carbosilane dendrimer nanotechnology outlines of the broad HIV blocker profile. *J. Control. Release* 161, 949–958. doi: 10.1016/j.jconrel.2012.04.050

Cole, L. E., Ross, R. D., Tilley, J. M., Vargo-Gogola, T., and Roeder, R. K. (2015). Gold nanoparticles as contrast agents in x-ray imaging and computed tomography. *Nanomedicine* 10, 321–341. doi: 10.2217/nnm.14.171

Combelles, C. M. H., Cekleniak, N. A., Racowsky, C., and Albertini, D. F. (2002). Assessment of nuclear and cytoplasmic maturation in in-vitro matured human oocytes. *Hum. Reprod.* 17, 1006–1016. doi: 10.1093/humrep/17.4.1006

Craighead, H. (2006). Future lab-on-a-chip technologies for interrogating individual molecules. *Nature* 442, 387–393. doi: 10.1038/nature05061

Cunha-Reis, C., Machado, A., Barreiros, L., Araújo, F., Nunes, R., Seabra, V., et al. (2016). Nanoparticles-in-film for the combined vaginal delivery of anti-HIV microbicide drugs. *J. Control. Release* 243, 43–53. doi: 10.1016/j.jconrel.2016.09.020

Das, J., Choi, Y. J., Song, H., and Ki, J. H. (2016). Potential toxicity of engineered nanoparticles in mammalian germ cells and developing embryos: treatment strategies and anticipated applications of nanoparticles in gene delivery. *Hum. Reprod. Update* 22, 588–619. doi: 10.1093/humupd/dmw020

De Almeida Borges, V. R., Da Silva, J. H., Barbosa, S. S., Nasciutti, L. E., Cabral, L. M., and De Sousa, V. P. (2016). Development and pharmacological evaluation of *in vitro* nanocarriers composed of lamellar silicates containing copaiba oil-resin for treatment of endometriosis. *Mater. Sci. Eng. C Mater. Biol. Appl.* 64, 310–317. doi: 10.1016/j.msec.2016.03.094

Duarah, S., Durai, R. D., and Narayanan, V. B. (2017). Nanoparticle-in-gel system for delivery of vitamin C for topical application. *Drug Deliv. Transl. Res.* 7, 750–760. doi: 10.1007/s13346-017-0398-z

Engelberth, S. A., Hempel, N., and Bergkvist, M. (2017). Cationic dendritic starch as a vehicle for photodynamic therapy and siRNA co-delivery. *J. Photochem. Photobiol. B Biol.* 168, 185–192. doi: 10.1016/j.jphotobiol.2017.02.013

Fauser, B. C. J. M., Devroey, P., and Macklon, N. S. (2005). Multiple birth resulting from ovarian stimulation for subfertility treatment. *Lancet* 365, 1807–1816. doi: 10.1016/S0140-6736(05)66478-1

Ferris, J., Mahboubi, K., MacLusky, N., King, W. A., and Favetta, L. A. (2016). BPA exposure during *in vitro* oocyte maturation results in dose-dependent alterations to embryo development rates, apoptosis rate, sex ratio and gene expression. *Reprod. Toxicol.* 59, 128–138. doi: 10.1016/j.reprotox.2015.12.002

Friedman, B. E., Davis, L. B., Lathi, R. B., Westphal, L. M., Baker, V. L., and Milki, A. A. (2011). Age-related success with elective single versus double blastocyst transfer. *ISRN Obstet. Gynecol.* 2011:656204. doi: 10.5402/2011/656204

Fujitani, T., Inomata, A., Ogata, A., Sakamoto, Y., Hirose, A., Nishimura, T., et al. (2015). Comparison of fetal toxicity of various multi-wall carbon nanotubes in mice. *Toxicol. Rep.* 2, 1404–1408. doi: 10.1016/j.toxrep.2015.07.014

Fulka, J. Jr., First, N. L., and Moor, R. M. (1998). Nuclear and cytoplasmic determinants involved in the regulation of mammalian oocyte maturation. *Mol. Hum. Reprod.* 4, 41–49. doi: 10.1093/molehr/4.1.41

Gasparotto, G., Costa, J. P. C., Costa, P. I., Zaghete, M. A., and Mazon, T. (2017). Electrochemical immunosensor based on ZnO nanorods-Au nanoparticles nanohybrids for ovarian cancer antigen CA-125 detection. *Mater. Sci. Eng. C* 76, 1240–1247. doi: 10.1016/j.msec.2017.02.031

Gaudet, D., Stroes, E. S., Méthot, J., Brisson, D., Tremblay, K., Sophie, J., et al. (2016). Long-term retrospective analysis of gene therapy with alipogene tiparvovec and its effect on lipoprotein lipase deficiency-induced pancreatitis. *Hum. Gene Ther.* 27, 916–925. doi: 10.1089/hum.2015.158

Goto, K., Kajihara, Y., Kosaka, S., Koba, M., Nakanishi, Y., and Ogawa, K. (1988). Pregnancies after co-culture of cumulus cells with bovine embryos derived from in-vitro fertilization of in-vitro matured follicular oocytes. *Reproduction* 83, 753–758. doi: 10.1530/jrf.0.0830753

Guan, S., and Rosenecker, J. (2017). Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems. *Gene Ther.* 24, 133–143. doi: 10.1038/gt.2017.5

Guo, W., Yang, W., Wang, Y., Sun, X., Liu, Z., Zhang, B., et al. (2014). Color tunable Gd-Zn-Cu-In-S/ZnS quantum dots for dual modality magnetic resonance and fluorescence imaging. *Nano Res.* 7, 1581–1591. doi: 10.1007/s12274-014-0518-8

Guo, X., Guo, N., Zhao, J., and Cai, Y. (2017). Active targeting co-delivery system based on hollow mesoporous silica nanoparticles for antitumor therapy in ovarian cancer stem-like cells. *Oncol. Rep.* 38, 1442–1450. doi: 10.3892/or.2017.5829

Gupta, P. R., and Huckefeldt, R. M. (2017). Gene therapy for inherited retinal degenerations: initial successes and future challenges. *J. Neural Eng.* 14:51002. doi: 10.1088/1741-2552/aa7a27

Hill, E. K., and Li, J. (2017). Current and future prospects for nanotechnology in animal production. *J. Anim. Sci. Biotechnol.* 8:26. doi: 10.1186/s40104-017-0157-5

Hua, X., Yang, Q., Dong, Z., Zhang, J., Zhang, W., Wang, Q., et al. (2017). Magnetically triggered drug release from nanoparticles and its applications in anti-tumor treatment. *Drug Deliv.* 24, 511–518. doi: 10.1080/10717544.2016.1256001

Huang, J. Y. J., Chian, R.-C., and Tan, S. L. (2010). Ovarian hyperstimulation syndrome prevention strategies: *in vitro* maturation. *Semin. Reprod. Med.* 28, 519–531. doi: 10.1055/s-0030-1265680

Hwu, Y. M., Lee, R. K., Chen, C. P., Su, J. T., Chen, Y. W., and Lin, S. P. (1998). Development of hatching blastocysts from immature human oocytes following in-vitro maturation and fertilization using a co-culture system. *Hum. Reprod.* 13, 1916–1921. doi: 10.1093/humrep/13.7.1916

Indrasekara, A. S. D. S., Paladini, B. J., Naczynski, D. J., Starovoytov, V., Moghe, P. V., and Fabris, L. (2013). Dimeric gold nanoparticle assemblies as tags for SERS-based cancer detection. *Adv. Healthc. Mater.* 2, 1370–1376. doi: 10.1002/adhm.201200370

Ishii, T. (2017). Reproductive medicine involving genome editing: clinical uncertainties and embryological needs. *Reprod. Biomed. Online* 34, 27–31. doi: 10.1016/j.rbmo.2016.09.009

Johari-Ahar, M., Rashidi, M. R., Barar, J., Aghaie, M., Mohammadnejad, D., Ramazani, A., et al. (2015). An ultra-sensitive impedimetric immunosensor for detection of the serum oncomarker CA-125 in ovarian cancer patients. *Nanoscale* 7, 3768–3779. doi: 10.1039/C4NR06687A

Karuputhula, N. B., Chattopadhyay, R., Chakravarty, B., and Chaudhury, K. (2013). Oxidative status in granulosa cells of infertile women undergoing IVF. *Syst. Biol. Reprod. Med.* 59, 91–98. doi: 10.3109/19396368.2012.743197

Kim, T. S., Lee, S. H., Gang, G. T., Lee, Y. S., Kim, S. U., Koo, D. B., et al. (2010). Exogenous DNA uptake of boar spermatozoa by a magnetic nanoparticle vector system. *Reprod. Domest. Anim.* 45, e201–e206. doi: 10.1111/j.1439-0531.2009.01516.x

Komninou, E. R., Remião, M. H., Lucas, C. G., Domingues, W. B., Basso, A. C., Jornada, D. S., et al. (2016). Effects of two types of melatonin-loaded nanocapsules with distinct supramolecular structures: polymeric (NC) and lipid-core nanocapsules (LNC) on bovine embryo culture model. *PLOS ONE* 11:e0157561. doi: 10.1371/journal.pone.0157561

Langbeen, A., De porte, H. F. M., Bartholomeus, E., Leroy, J. L., and Bols, P. E. J. (2015). Bovine *in vitro* reproduction models can contribute to the development of (female) fertility preservation strategies. *Theriogenology* 84, 477–489. doi: 10.1016/j.theriogenology.2015.04.009

Li, F., Li, Y., Feng, J., Dong, Y., Wang, P., Chen, L., et al. (2017). Ultrasensitive amperometric immunosensor for PSA detection based on Cu2O@CeO2-Au nanocomposites as integrated triple signal amplification strategy. *Biosens. Bioelectron.* 87, 630–637. doi: 10.1016/j.bios.2016.09.018

Li, W. J., Zhou, X. L., Liu, B. L., Dai, J. J., Song, P., and Teng, Y. (2016). Effect of nanoparticles on the survival and development of vitrified porcine GV oocytes. *Cryo Lett.* 37, 401–405.

Liang, P., Xu, Y., Zhang, X., Ding, C., Huang, R., Zhang, Z., et al. (2015). CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein Cell* 6, 363–372. doi: 10.1007/s13238-015-0153-5

Lin, M., Gao, Y., Diefenbach, T. J., Shen, J. K., Hornicek, F. J., Park, Y. I., et al. (2017). Facial layer-by-layer engineering of upconversion nanoparticles for gene delivery: near-infrared-initiated fluorescence resonance energy transfer tracking and overcoming drug resistance in ovarian cancer. *ACS Appl. Mater. Interfaces* 9, 7941–7949. doi: 10.1021/acsami.6b15321

Liu, Q., Ma, P., Liu, L., Ma, G., Ma, J., Liu, X., et al. (2017). Evaluation of PLGA containing anti-CTLA4 inhibited endometriosis progression by regulating CD4 + CD25 + Treg cells in peritoneal fluid of mouse endometriosis model. *Eur. J. Pharm. Sci.* 96, 542–550. doi: 10.1016/j.ejps.2016.10.031

Liu, Z., Cai, W., He, L., Nakayama, N., Chen, K., Sun, X., et al. (2007). In vivo biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. *Nat. Nanotechnol.* 2, 47–52. doi: 10.1038/nnano.2006.170

Lonergan, P., Rizos, D., Gutierrez-Adán, A., Moreira, P. M., Pintado, B., de la Fuente, J., et al. (2003). Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage *in vitro* or in vivo. *Biol. Reprod.* 69, 1424–1431. doi: 10.1095/biolreprod.103.018168

Lucas, C. G., Remião, M. H., Bruinsmann, F. A., Lopes, I. A. R., Borges, M. A., Feijó, A. L. S., et al. (2017). High doses of lipid-core nanocapsules do not affect bovine embryonic development *in vitro*. *Toxicol. In Vitro* 45(Pt 1), 194–201. doi: 10.1016/j.tiv.2017.09.013

Lucas, C. G., Remião, M. H., Komninou, E. R., Domingues, W. B., Haas, C., Leon, P. M., et al. (2015). Tretinoin-loaded lipid-core nanocapsules decrease reactive oxygen species levels and improve bovine embryonic development during *in vitro* oocyte maturation. *Reprod. Toxicol.* 58, 131–139. doi: 10.1016/j.reprotox.2015.10.004

Ma, H., Marti-Gutierrez, N., Park, S.-W., Wu, J., Lee, Y., Suzuki, K., et al. (2017). Correction of a pathogenic gene mutation in human embryos. *Nature* 548, 413–419. doi: 10.1038/nature23305

Makhluf, S. B.-D., Abu-Mukh, R., Rubinstein, S., Breitbart, H., and Gedanken, A. (2008). Modified PVA-Fe3O4 nanoparticles as protein carriers into sperm cells. *Small* 4, 1453–1458. doi: 10.1002/smll.200701308

Manconi, M., Marongiu, F., Manca, M. L., Caddeo, C., Sarais, G., Cencetti, C., et al. (2017). Nanoincorporation of bioactive compounds from red grape pomaces: *in vitro* and ex vivo evaluation of antioxidant activity. *Int. J. Pharm.* 523, 159–166. doi: 10.1016/j.ijpharm.2017.03.037

Mancuso, A. C., Boulet, S. L., Duran, E., Munch, E., Kissin, D. M., and Van Voorhis, B. J. (2016). Elective single embryo transfer in women less than age 38 years reduces multiple birth rates, but not live birth rates, in United States fertility clinics. *Fertil. Steril.* 106, 1107–1114. doi: 10.1016/j.fertnstert.2016.06.017

Mao, L., Lou, H., Lou, Y., Wang, N., and Jin, F. (2014). Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. *Reprod. Biomed. Online* 28, 284–299. doi: 10.1016/j.rbmo.2013.10.016

Martina, M. S., Fortin, J. P., Ménager, C., Clément, O., Barratt, G., Grabielle-Madelmont, C., et al. (2005). Generation of superparamagnetic liposomes revealed as highly efficient MRI contrast agents for in vivo imaging. *J. Am. Chem. Soc.* 127, 10676–10685. doi: 10.1021/ja0516460

Melnik, E. A., Buzulukov, Y. P., Demin, V. F., Demin, V. A., Gmoshinski, I. V., Tyshko, N. V., et al. (2013). Transfer of silver nanoparticles through the placenta and breast milk during in vivo experiments on rats. *Acta Nat.* 5, 107–115.

Mersereau, J., Stanhiser, J., Coddington, C., Jones, T., Luke, B., and Brown, M. B. (2017). Patient and cycle characteristics predicting high pregnancy rates with single-embryo transfer: an analysis of the Society for Assisted Reproductive Technology outcomes between 2004 and 2013. *Fertil. Steril.* 108, 750–756. doi: 10.1016/j.fertnstert.2017.07.1167

Miyake, Y., Ishikawa, S., Kimura, Y., Son, A., Imai, H., Matsuda, T., et al. (2015). Pharmacokinetics of chiral dendrimer-triamine-coordinated Gd-MRI contrast agents evaluated by in vivo MRI and estimated by *in vitro* QCM. *Sensors* 15, 31973–31986. doi: 10.3390/s151229900

Morris, E. C., Fox, T., Chakraverty, R., Tendeiro, R., Snell, K., Rivat, C., et al. (2017). Gene therapy for Wiskott-Aldrich syndrome in a severely affected adult. *Blood* 130, 1327–1335. doi: 10.1182/blood-2017-04-777136

Mukundan, S., Ghaghada, K. B., Badea, C. T., Kao, C. Y., Hedlund, L. W., Provenzale, J. M., et al. (2006). A liposomal nanoscale contrast agent for preclinical CT in mice. *AJR Am. J. Roentgenol.* 186, 300–307. doi: 10.2214/AJR.05.0523

Naserzadeh, P., Ghanbary, F., Ashtari, P., Seydi, E., Ashtari, K., and Akbari, M. (2017). Biocompatibility assessment of titanium dioxide nanoparticles in mice feto- placental unit. *J. Biomed. Mater. Res. A* 106, 580–589. doi: 10.1002/jbm.a.36221

Nathwani, A. C., Davidoff, A. M., and Tuddenham, E. G. D. (2017). Advances in gene therapy for Haemophilia. *Hum. Gene Ther.* 28, 1004–1012. doi: 10.1089/hum.2017.167

Negre, O., Eggimann, A.-V., Beuzard, Y., Ribeil, J.-A., Bourget, P., Borwornpinyo, S., et al. (2016). Gene therapy of the β-hemoglobinopathies by lentiviral transfer of the β A(T87Q) - globin gene. *Hum. Gene Ther.* 27, 148–165. doi: 10.1089/hum.2016.007

Niemann, H., and Wrenzycki, C. (2000). Alterations of expression of developmentally important genes in preimplantation bovine embryos by *in vitro* culture conditions: implications for subsequent development. *Theriogenology* 53, 21–34. doi: 10.1016/S0093-691X(99)00237-X

Ortega-Hrepich, C., Stoop, D., Guzmán, L., Van Landuyt, L., Tournaye, H., Smitz, J., et al. (2013). A "freeze-all" embryo strategy after *in vitro* maturation: a novel approach in women with polycystic ovary syndrome? *Fertil. Steril.* 100, 1002–1007. doi: 10.1016/j.fertnstert.2013.06.018

Ozsoz, M., Erdem, A., Kerman, K., Ozkan, D., Tugrul, B., Topcuoglu, N., et al. (2003). Electrochemical genosensor based on colloidal gold nanoparticles for the detection of factor V leiden mutation using disposable pencil graphite electrodes. *Anal. Chem.* 75, 2181–2187. doi: 10.1021/ac026212r

Pal, M., and Khan, R. (2017). Graphene oxide layer decorated gold nanoparticles based immunosensor for the detection of prostate cancer risk factor. *Anal. Biochem.* 536, 51–58. doi: 10.1016/j.ab.2017.08.001

Pal, M. K., Rashid, M., and Bisht, M. (2015). Multiplexed magnetic nanoparticle-antibody conjugates (MNPs-ABS) based prognostic detection of ovarian cancer biomarkers, CA-125, β-2M and ApoA1 using fluorescence spectroscopy with comparison of surface plasmon resonance (SPR) analysis. *Biosens. Bioelectron.* 73, 146–152. doi: 10.1016/j.bios.2015.05.051

Poels, J., Abou-Ghannam, G., Decamps, A., Leyman, M., Rieux, A. D., and Wyns, C. (2016). Transplantation of testicular tissue in alginate hydrogel loaded with VEGF nanoparticles improves spermatogonial recovery. *J. Control. Release* 234, 79–89. doi: 10.1016/j.jconrel.2016.05.037

Pradhan, L., Thakur, B., Srivastava, R., Ray, P., and Bahadur, D. (2016). Assessing therapeutic potential of magnetic mesoporous nanoassemblies for chemo-resistant tumors. *Theranostics* 6, 1557–1572. doi: 10.7150/thno.15231

Presnova, G., Presnov, D., Krupenin, V., Grigorenko, V., Trifonov, A., Andreeva, I., et al. (2017). Biosensor based on a silicon nanowire field-effect transistor functionalized by gold nanoparticles for the highly sensitive determination of prostate specific antigen. *Biosens. Bioelectron.* 88, 283–289. doi: 10.1016/j.bios.2016.08.054

Qi, W., Bi, J., Zhang, X., Wang, J., Wang, J., Liu, P., et al. (2014). Damaging effects of multi-walled carbon nanotubes on pregnant mice with different pregnancy times. *Sci. Rep.* 4:4352. doi: 10.1038/srep04352

Remião, M. H., Lucas, C. G., Domingues, W. B., Silveira, T., Barther, N. N., Komninou, E. R., et al. (2016). Melatonin delivery by nanocapsules during *in vitro* bovine oocyte maturation decreased the reactive oxygen species of oocytes and embryos. *Reprod. Toxicol.* 63, 70–81. doi: 10.1016/j.reprotox.2016.05.016

Riley, M., and Vermerris, W. (2017). Recent advances in nanomaterials for gene delivery—a review. *Nanomaterials* 7:E94. doi: 10.3390/nano7050094

Roberts, C. M., Shahin, S. A., Wen, W., Finlay, J. B., Dong, J., Wang, R., et al. (2017). Nanoparticle delivery of siRNA against TWIST to reduce drug resistance and tumor growth in ovarian cancer models. *Nanomedicine* 13, 965–976. doi: 10.1016/j.nano.2016.11.010

Romek, M., Gajda, B., Krzysztofowicz, E., Kucia, M., Uzarowska, A., and Smorag, Z. (2017). Improved quality of porcine embryos cultured with hyaluronan due to the modification of the mitochondrial membrane potential and reactive oxygen species level. *Theriogenology* 102, 1–9. doi: 10.1016/j.theriogenology.2017.06.026

Salzano, A., Albero, G., Zullo, G., Neglia, G., Abdel-Wahab, A., Bifulco, G., et al. (2014). Effect of resveratrol supplementation during culture on the quality and cryotolerance of bovine *in vitro* produced embryos. *Anim. Reprod. Sci.* 151, 91–96. doi: 10.1016/j.anireprosci.2014.09.018

Selokar, N. L., Saini, M., Agrawal, H., Palta, P., Chauhan, M. S., Manik, R., et al. (2015). Downregulation of DNA methyltransferase 1 in zona-free cloned buffalo (*Bubalus bubalis*) embryos by small interefering RNA improves *in vitro* development but does not alter DNA methylation level. *Cell. Reprogram.* 17, 89–94. doi: 10.1089/cell.2014.0056

Shalaby, S. M., Khater, M. K., Perucho, A. M., Mohamed, S. A., Helwa, I., Laknaur, A., et al. (2016). Magnetic nanoparticles as a new approach to improve the efficacy of gene therapy against differentiated human uterine fibroid cells and tumor-initiating stem cells. *Fertil. Steril.* 105, 1638–1648. doi: 10.1016/j.fertnstert.2016.03.00

Shi, H., Xia, T., Nel, A. E., and Yeh, J. I. (2007). Part II: coordinated biosensors - development of enhanced nanobiosensors for biological and medical applications. *Nanomedicine* 2, 599–614. doi: 10.2217/17435889.2.5.599

Sillerud, L. O. (2016). Quantitative [Fe]MRI of PSMA-targeted SPIONs specifically discriminates among prostate tumor cell types based on their PSMA expression levels. *Int. J. Nanomedicine* 11, 357–371. doi: 10.2147/IJN.S93409

Sinclair, K. D. (2008). Assisted reproductive technologies and pregnancy outcomes: mechanistic insights from animal studies. *Semin. Reprod. Med.* 26, 153–161. doi: 10.1055/s-2008-1042954

Singh, A. K., Chakravarty, B., and Chaudhury, K. (2015). Nanoparticle-assisted combinatorial therapy for effective treatment of endometriosis. *J. Biomed. Nanotechnol.* 11, 789–804. doi: 10.1166/jbn.2015.2020

Sriraman, S. K., Pan, J., Sarisozen, C., Luther, E., and Torchilin, V. (2016). Enhanced cytotoxicity of folic acid-targeted liposomes co-loaded with C6 ceramide and doxorubicin: *in vitro* evaluation on HeLa, A2780-ADR, and H69-AR Cells. *Mol. Pharm.* 13, 428–437. doi: 10.1021/acs.molpharmaceut.5b00663

Stoeva, S. I., Lee, J. S., Thaxton, C. S., and Mirkin, C. A. (2006). Multiplexed DNA detection with biobarcoded nanoparticle probes. *Angew. Chem. Int. Ed. Engl.* 45, 3303–3306. doi: 10.1002/anie.200600124

Sun, W., Ji, W., Hall, J. M., Hu, Q., Wang, C., Beisel, C. L., et al. (2015). Efficient delivery of CRISPR-Cas9 for genome editing via self- assembled DNA nanoclews. *Angew. Chem. Int. Ed. Engl.* 54, 12029–12033. doi: 10.1002/anie.201506030

Tamura, H., Takasaki, A., Miwa, I., Taniguchi, K., Maekawa, R., Asada, H., et al. (2008). Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J. Pineal Res.* 44, 280–287. doi: 10.1111/j.1600-079X.2007.00524.x

Tannus, S., Hatirnaz, S., Tan, J., Ata, B., Tan, S.-L., Hatirnaz, E., et al. (2017). Predictive factors for live birth after *in vitro* maturation of oocytes in women with polycystic ovary syndrome. *Arch. Gynecol. Obstet.* doi: 10.1007/s00404-017-4561-z [Epub ahead of print].

Thaxton, C. S., Elghanian, R., Thomas, A. D., Stoeva, S. I., Lee, J.-S., Smith, N. D., et al. (2009). Nanoparticle-based bio-barcode assay redefines "undetectable" PSA and biochemical recurrence after radical prostatectomy. *Proc. Natl. Acad. Sci. U.S.A.* 106, 18437–18442. doi: 10.1073/pnas.0904719106

Todd, T., Zhen, Z., Tang, W., Chen, H., Wang, G., Chuang, Y.-J., et al. (2014). Iron oxide nanoparticle encapsulated diatoms for magnetic delivery of small molecules to tumors. *Nanoscale* 6, 2073–2076. doi: 10.1039/c3nr05623f

Truong, T. T., Soh, Y. M., and Gardner, D. K. (2016). Antioxidants improve mouse preimplantation embryo development and viability. *Hum. Reprod.* 31, 1445–1454. doi: 10.1093/humrep/dew098

Tsirigotis, M. (1998). Blastocyst stage transfer: pitfalls and benefits. Too soon to abandon current practice? *Hum. Reprod.* 13, 3285–3289. doi: 10.1093/humrep/13.12.3285

Vittorio, O., Duce, S. L., Pietrabissa, A., and Cuschieri, A. (2011). Multiwall carbon nanotubes as MRI contrast agents for tracking stem cells. *Nanotechnology* 22:95706. doi: 10.1088/0957-4484/22/9/095706

Vulliemoz, N. R., McVeigh, E., and Kurinczuk, J. (2012). *in vitro* fertilisation: perinatal risks and early childhood outcomes. *Hum. Fertil.* 15, 62–68. doi: 10.3109/14647273.2012.663571

Wang, F., Tian, X., Zhang, L., Tan, D., Reiter, R. J., and Liu, G. (2013). Melatonin promotes the *in vitro* development of pronuclear embryos and increases the efficiency of blastocyst implantation in murine. *J. Pineal Res.* 55, 267–274. doi: 10.1111/jpi.12069

Wang, F., Tian, X., Zhou, Y., Tan, D., Zhu, S., Dai, Y., et al. (2014). Melatonin improves the quality of *in vitro* produced (IVP) bovine embryos: implications for blastocyst development, cryotolerance, and modifications of relevant gene expression. *PLOS ONE* 9:e93641. doi: 10.1371/journal.pone.0093641

Yao, H. C., Su, L., Zeng, M., Cao, L., Zhao, W. W., Chen, C. Q., et al. (2016). Construction of magnetic-carbon-quantum-dots-probe-labeled apoferritin nanocages for bioimaging and targeted therapy. *Int. J. Nanomedicine* 11, 4423–4438. doi: 10.2147/IJN.S108039

Yin, Y., Chen, G., Li, K., Liao, Q., Zhang, S., Ma, N., et al. (2017). Propensity score-matched study and meta-analysis of cumulative outcomes of day 2/3 versus day 5/6 embryo transfers. *Front. Med.* 11, 563–569. doi: 10.1007/s11684-017-0535-6

Yuan, J., Duan, R., Yang, H., Luo, X., and Xi, M. (2012). Detection of serum human epididymis secretory protein 4 in patients with ovarian cancer using a label-free biosensor based on localized surface plasmon resonance. *Int. J. Nanomedicine* 7, 2921–2928. doi: 10.2147/IJN.S32641

Zegers-Hochschild, F., Adamson, G. D., Dyer, S., Racowsky, C., de Mouzon, J., Sokol, R., et al. (2017). The international glossary on infertility and fertility care, 2017. *Fertil. Steril.* 108, 393–406. doi: 10.1016/j.fertnstert.2017.06.005

Zhao, M. D., Cheng, J. L., Yan, J. J., Chen, F. Y., Sheng, J. Z., Sun, D. L., et al. (2016). Hyaluronic acid reagent functional chitosan-PEI conjugate with AQP2-siRNA suppressed endometriotic lesion formation. *Int. J. Nanomedicine* 11, 1323–1336. doi: 10.2147/IJN.S99692

Zhao, M. D., Sun, Y. M., Fu, G. F., Du, Y. Z., Chen, F. Y., Yuan, H., et al. (2012). Gene therapy of endometriosis introduced by polymeric micelles with glycolipid-like structure. *Biomaterials* 33, 634–643. doi: 10.1016/j.biomaterials.2011.09.077

Zhu, X., Li, J., He, H., Huang, M., Zhang, X., and Wang, S. (2015). Application of nanomaterials in the bioanalytical detection of disease-related genes. *Biosens. Bioelectron.* 74, 113–133. doi: 10.1016/j.bios.2015.04.069

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Liposome-Encapsulated Baicalein Suppressed Lipogenesis and Extracellular Matrix Formation in Hs68 Human Dermal Fibroblasts

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The dermis of human skin contains large numbers of fibroblasts that are responsible for the production of the extracellular matrix (ECM) that supporting skin integrity, elasticity and wound healing. Previously, an *in vivo* study demonstrated that dermal fibroblasts siting in the lower dermis are capable to convert into skin adipose layer and hence fibroblast lipogenesis may vary the structure and elasticity of dermis. In the present study, Hs68 human dermal fibroblasts were utilized as an *in vitro* model to study the lipogenesis via using adipogenic differentiation medium (ADM). Baicalein, isolated from *Scutellaria baicalensis*, is one of the flavonoids to inhibit adipocyte differentiation due to high antioxidant activity *in vitro*. In order to develop a suitable formulation for baicalein (a poorly water-soluble drug), soybean phosphatidylcholine (SPC) was used to prepare baicalein-loaded liposomes to enhance drug bioavailability. Our results demonstrated that liposome-encapsulated baicalein protected cell viability and increased cellular uptake efficiency of Hs68 fibroblasts. Lipid accumulation, triglyceride synthesis and gene expressions of lipogenesis enzymes (FABP4 and LPL) were significantly increased in ADM-stimulated Hs68 fibroblasts but subsequently suppressed by liposome-encapsulated baicalein. In addition, ADM-induced TNF-α expression and related inflammatory factors was down-regulated by liposome-encapsulated baicalein. Through ADM-induced lipogenesis, the protein expression of elastin, type I and type III collagens increased remarkably, whereas liposome-encapsulated baicalein can down-regulate ADM-induced ECM protein synthesis. Taken together, we found that liposome-encapsulated baicalein can inhibit ADM-induced lipid accumulation and ECM formation in Hs68 fibroblasts through the suppression of lipogenesis enzymes and inflammatory responses. Liposome-encapsulated baicalein may have the potential to improve wound healing and restore skin structure after skin injury.

Keywords: baicalein, liposomes, Hs68 human dermal fibroblast, adipogenic differentiation medium, lipogenesis

INTRODUCTION

Dermis in skin is the layer lies between epidermis and subcutaneous adipose tissue. Dermis is composed of fibrous and elastic tissue, thereby providing strength and flexibility to skin. Dermis is the thickest layer of the skin, but that can be varied based on body mass index (BMI), gender and locations. Recently, many researches revealed the relationship between adipose tissue in correlation with dermal fibroblasts. Ezure and Amano (2010) and Hew et al. (2016) demonstrated that the subcutaneous adipose layer of mice was remarkably thickened after being fed with high-fat diet, while the dermal layer was thinned (Ezure and Amano, 2010; Hew et al., 2016). Hence, such increase of adipose tissue may reduce the proliferation of dermal fibroblasts and elasticity of skin (Ezure and Amano, 2010). Moreover, adipogenesis-defect animal model indicated that intradermal adipocytes could mediate fibroblast recruitment during skin wound healing (Schmidt and Horsley, 2013). Interestingly, Wojciechowicz et al. (2013) confirmed that dermal fibroblasts siting in the lower dermis are capable to convert into skin adipose layer independently without influence from subcutaneous adipose tissue. Furthermore, the thickness of the lower dermis increased was found concomitant with the extension and downgrowth of hair follicles. Therefore, a key objective of studying the lipogenesis in Hs68 fibroblasts is to determine if and how lipogenesis can modulate inflammatory responses and change extracellular matrix (ECM) structure and composition in human dermal fibroblasts.

Dermal fibroblasts are the major cell population in dermis which are responsible for ECM production and wound healing. In the study reported by Rakar et al. (2012) primary human dermal fibroblasts were found to differentiate toward adipocytes, osteoblasts and chondrocytes using different induction media. Hence, relatively undifferentiated fibroblasts can express a particular phenotype depend on physiological stimuli and microenvironmental factors to which they are exposed (Koumas et al., 2003). This discovery supports the concept of fibroblast plasticity and proposes that fibroblasts can be transformed into adipocytes. In previous report suing human neonatal and adult lung tissues, alveolar interstitial fibroblasts were detected with the presence of lipid and the expression of adipocyte differentiation-related protein (ADRP), a protein necessary for lipid uptake, leading to their classification as lipid-containing fibroblasts or lipofibroblasts (Rehan et al., 2006). Lung specimens harvesting from patients with idiopathic pulmonary fibrosis (IPF) were found to have decreased lipofibroblast marker expression compared with non-IPF control samples (Bhattacharya, 2016), resulting in a hypothesis that conversion of fibroblasts into lipid-containing cells or lipofibroblasts may be able to prevent tissue fibrosis, abnormal wound healing, and hypertrophic scars. However, in skin wound healing, the role of lipogenesis in dermal fibroblasts were under investigated and the mechanisms has yet to be defined. Our previous study confirmed that adipogenic differentiation medium (ADM) can stimulate the differentiation of Hs68 human dermal fibroblasts to adipocyte-like cells through the lipid accumulation and mRNA expressions of PPAR-γ, LPL and FABP4 (Fang et al., 2016). In the present study, we aim to further investigate the impact of lipogenesis on the inflammation responses and ECM formation in Hs68 fibroblasts.

Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one), one of the remarkable flavonoids, is isolated from the root of *Scutellaria baicalensis*. Baicalein possesses a variety of biological activities, including high antioxidant, anti-inflammatory, anti-proliferative, anti-apoptotic and anti-tumor activities (Gao et al., 2013). Baicalein has been demonstrated to decrease skin thickness and to suppress the expression levels of matrix metalloproteinase (MMP)-9, and vascular endothelial growth factor (VEGF) in ultraviolet (UV) B irradiated skin of mice models (Kimura and Sumiyoshi, 2011). Moreover, baicalein is known to inhibit radiation induced expression of nuclear transcription factor nuclear factor kappa B (NF-κB) and Cyclooxygenase-2 (COX-2) in human keratinocytes (Kimura and Sumiyoshi, 2011). Seo et al. (2014) found that baicalein can inhibit lipid accumulation and adipocyte differentiation by suppressing adipogenic factors such as PPARγ and C/EBPα through m-TOR signaling pathway in 3T3-L1 fibroblasts. However, using baicalein in therapeutic application are limited due to its low water solubility and poor oral bioavailability (Huang et al., 2014; de Oliveira et al., 2015). Recently, Rajkumari et al. (2017) have used baicalein as a reducing and capping agent in the synthesis of gold nanoparticles to inhibit Pseudomonas aeruginosa PAO1 biofilm formation. Li X. et al. (2017) also synthesized amine-modified mesoporous silica nanoparticles to encapsulate baicalein to exhibit anti-inflammatory effect on primary human gingival epithelial cells. Interestingly, baicalein was found to reduce the cytotoxicity of ZnO nanoparticles in Caco-2 cells (Li Y. et al., 2017). Therefore, liposomal nanoencapsulation of baicalein was introduced and investigated aiming to improve therapeutic efficacy through the increase of drug solubility and cell absorption efficiency (Moulaoui et al., 2015)

Composite phospholipid liposomes, similar to the lipid bilayer of cell membrane, have been formulated in nano-scaled sizes to increase the *in vivo* bioavailability for hydrophobic drugs (Kalepu and Nekkanti, 2015; Ong et al., 2016). Liposomes can be modified with various lipids to enhance drug loading efficiency and release characteristics (Mohammed et al., 2004) with reduced cytotoxicity, better biocompatibility and stability (Zeng et al., 2016). Various techniques, such as Bangham method, the detergent depletion method, the ether/ethanol injection method, the reverse phase evaporation and the emulsion method are previously reported to formulate drug-loaded liposomes with high entrapment efficiency (EE), narrow polydiversity index (PDI) and long term stability (Galović Rengel et al., 2002; Bergstrand et al., 2003). Based on low aqueous solubility and high cytotoxicity of baicalein on Hs 68 human dermal fibroblasts, phosphatidylcholine (PC) -based liposomes were used to encapsulate baicalein. Thereafter, we investigated the inhibitory effects of liposome-encapsulated baicalein on the ADM-induced adipogenesis, inflammatory responses and ECM synthesis in human dermal fibroblasts, Hs68.

MATERIALS AND METHODS

Chemicals and Cell Culture

Baicalein was purchased from Sigma-Aldrich, USA. Phospholipon 90G (phosphatidylcholine 90%) was acquired from American Lecithin Company, Germany. All cell culture materials including, Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, adipocyte differentiation medium (ADM) were obtained from Gibco (Grand Island, NY, United States). Oil red o staining kit was purchased from Lifeline Cell Technology (Frederick, MD, United States). Adipogenesis assay kit was purchased from Sigma–Aldrich (St. Louis, MO, United States). All reagents and solvents are for research use only. Human foreskin fibroblasts (Hs68) were obtained from ATCC (Manassas, VA, United States) and Murine macrophages RAW 264.7 were purchased from Bioresource collection and Research Center, Food Industry Research and Development Institute, Taiwan. Both cell lines were cultured in DMEM supplemented with 10% v/v FBS, 100 units/ml penicillin and 100 μg/ml of streptomycine under steady state condition at 37°C with 5% CO_2 in a humidified incubator.

Liposomal Formulation

Baicalein-loaded liposomes were generated using a thin-film hydration and size reduction procedure as previously described (Yeh et al., 2015). Briefly, 100 mg of phospholipids were dissolved in 8 ml of chloroform, series amounts (20–60 μg) of baicalein were dissolved in 2 ml of ethanol and mixed together in a round-bottom flask. The organic solvents were evaporated by using rotary evaporator (Eyela, N-1000, Japan) at 45°C prior to vacuum dry to form a dry lipid film. The lipid film was rehydrated by addition of 2 ml PBS (Phosphate buffered saline). Liposomes were then resized and uniformed through extruding polycarbonate membranes with series decreased pore sizes from 400 nm, 200 nm to 100 nm (Avanti Mini Extruderm, Alabaster, AL, United States). Empty liposomes were prepared by the same process with drug-free methanol.

Particle Characterization

The particle stability of liposomes was identified by storing and measuring the particle sizes over 2 weeks. The particle sizes were measured by using a Dynamic Light Scattering Instrument (HORIBA, LB-550, Japan). The solution of baicalein-loaded liposomes was diluted in approximate 30 times with double-distilled water to make sure that the light scattering intensity was in the instrument's detectable range. The Polydispersity index (PDI) and zeta potential of liposomes were determined by Dynamic Light Scattering Analyzer (Malvern, Malvern Nano-Zs, England).

Entrapment Efficiency

Loading efficiency of baicalein in liposomes was assessed using a high-speed centrifugation once the liposomes were formulated. Baicalein-loaded liposomes were spun down at 80,000 rpm by Beckman ultra-high speed centrifuge for 30 min. Next, the supernatants were carefully discarded and pellets were subsequently redissolved in the equal volume of ethanol. The concentration of baicalein in liposomes were then measured using an ELISA reader (Tecan, infinite M200) at wavelength of 277 nm. The encapsulation efficiency of baicalein in liposomes was calculated from the standard curve. The entrapment efficiency (EE) was obtained by the following formulation:

$$\text{EE\%} = \frac{\text{the amount of baicalein in liposomes}}{\text{initial amount of baicalein for drug loading}} \times 100\%$$

Cell Uptake of Baicalein-Loaded Liposomes

Cell uptake of baicalein-loaded liposomes in Hs68 human foreskin fibroblasts was examined using the fluorescent microscopy. DiI solution (1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate, 10 mg/ml, 1 μl) was added into liposomal solution to form DiI-loaded liposomes. Hs68 fibroblasts were cultured in 6 cm dishes at a density of 3×10^5 cells/ dish overnight at 37°C with 5% CO_2. After 1 day culture, cell culture medium was removed and replaced with DiI-loaded liposomes at various time intervals (0–24 h) prior to washing with PBS and fixation with 0.075% (v/v) formaldehyde solution for 30 min. In the control group, Hs68 fibroblasts were treated with DiI solution for 24 h. After fixation, cells were secondary washed with PBS and stained nuclei using DAPI solution (10 μg/ml) for 10 min. Finally, Hs68 fibroblasts were rinsed and mounted with PBS and photographed by microscope (Nikon TI-E) and CCD camera system (SPOT RT3). Fluorescent photographs were quantitative analyzed using Image J software (NIH, United State). Cell uptake efficiency was determined via measuring DiI fluorescence intensity.

Cell Viability

Human foreskin fibroblasts, Hs68 and murine macrophages, RAW264.7 were seeded at a density of 10^4 cells/well and 5×10^4 cells/well in 96 well plates individually for cell viability analysis. After incubation overnight, cells were then treated with pure baicalein, empty or baicalein-loaded liposomes in concentrations of 10 or 20 μg/ml respectively for 24 h. After baicalein treatment, cell culture medium were replaced with 200 μl of 100 μg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent and incubated for 4 h before measuring absorbance at 570 nm using ELISA reader (Tecan, infinite M200). Relative cell viability was demonstrated by percentage compared with control.

Nitrite Assay

RAW264.7 macrophages were seeded at a density of 5×10^5 cells/well in 24 well plates, following by treatment with pure baicalein, empty or baicalein-loaded liposomes, in concentrations of 10 and 20 μg/ml respectively for 24 h. After removing of supernatants, no-phenol red medium (200 μl) were added for 8 h of incubation. The release of nitric oxide from inflamed macrophages was measured by determining nitrite concentration. Nitrite-contained medium (100 μl) were mixed with 100 μl of griess reagents in 96 well plates. Absorbance was measured at wavelength of 550 nm using ELISA reader (Tecan,

infinite M200) after 15 min of shaking in dark. The reference values of nitric oxide were shown as the mean percentage of absorbance and standard deviation in comparison with lipopolysaccharide (LPS)-treated cells from two independent experiments.

Oil Red O Staining

Hs68 fibroblasts were seeded at a density of 10^5 cells/ dish in 6 cm dishes. After 24 h incubation, the culture medium was replenished with 20% (v/v) ADM and cultured for 14 days to induce lipogenesis, while pure baicalein, empty or baicalein-loaded liposomes were added at day 7. At the end of 14 days culture, cell culture medium was removed and rinsed with PBS twice. For histochemical examination, intracellular accumulation of lipid in Hs68 fibroblasts was performed using the Oil-red O staining kit (Lifeline Cell Technology, Carlsbad, CA, United States). Concisely, cells were fixed with 4% (v/v) paraformaldehyde fixative solution for 30 min, rinsed with PBS and incubated with 100% 1, 2-propanediol dehydration solution for 10 min at room temperature. Following by fixation and dehydration, oil red O stain solution was added and incubated at 37°C for 30 min prior to imaging of cell morphology under microscope (Nikon TI-E) and CCD camera system (SPOT RT3). Finally, 85% (v/v) 1,2-propanediol stain differential solution was added for 1 min to differentiate stain and the absorbance was measured by ELISA reader (Tecan, infinite M200) at the wavelength of 520 nm.

Triglyceride Assay

Quantitative analysis of triglyceride content in ADM-induced Hs68 fibroblasts was conducted using an adipogenesis assay kit (Sigma–Aldrich) according to the manufacturer's instructions. Hs68 fibroblasts were cultured and adipogenesis was induced as described above. Free drug, empty liposomes or baicalein-loaded liposomes were added to cells respectively at day 7. After 21 days of incubation, 100 μl of lipid extraction buffer was added and incubated for 30 min at 90°C. Cell medium became cloudy and then cooled down at room temperature. Cell medium was shaken for 1 min for homogenization followed by transfering 5–50 μl of lipid to another 96 well plate which were filled with adipogenesis assay buffer up to a total volume of 50 μl. Lipase solution (2 μl) was added and incubated for 10 min at room temperature for the degradation of triglyceride, following by adding master reaction mix for reaction of 30 min before measuring the absorbance at 570 nm using the ELISA reader (Tecan, infinite M200). Triglyceride content in Hs68 fibroblasts was calculated from the triglyceride (triolein)-equivalent standard curve.

Fluorescent Antibody Technique

Hs68 fibroblasts were seeded at a density of 10^5 cells/dish and treated with ADM for 14 days in the presence or absence of liposomal samples. After 14 days of adipogenic differentiation, medium was removed and rinsed with PBS. Cells were fixed with 10% formaldehyde at 4°C for 30 min and washed with PBS twice. 0.1% Nonidet P-40 in PBS was added and incubated at room temperature for 10 min. After discarding Nonidet P-40 solution, cells were washed with PBS twice and blocked with BSA solution (2% w/v in PBS) for 30 min. Thereafter, the primary antibodies of anti-collagen I IgG produced in rabbit (1:500 in PBS, ab34710, Abcam, Cambridge, United Kingdom), anti-collagen III IgG produced in mouse (1:1000 in PBS, ab6310, Abcam, Cambridge, United Kingdom), and anti-elastin IgG produced in rabbit (1:200 in PBS, ab21610, Abcam, Cambridge, United Kingdom) were added separately and kept at 4°C for overnight. After washing with PBS twice, the secondary antibody of H&L Dylight 594 anti-mouse IgG produced in goat (1:50 in PBS, ab96881 Abcam, Cambridge, United Kingdom) conjugated with reddish fluorescein (FITC) and green fluorescent anti-rabbit IgG produced in goat (1:200 in PBS, 111-095-003, Jackson ImmunoResearch Laboratories, WestGrove, PA, United States) were added in turn for 1 h of incubation. Finally, cells were washed with PBS twice and DAPI (10 μg/ml) was used to stain the nucleus of cells. The cell morphology was photographed by fluorescent microscopy equipped with CCD camera system (SPOT RT3). For quantitative analysis of elastin, type I and III collagen in hs68 fibroblasts, we used Image J software (NIH) to measure the fluorescence intensity in 10X images obtained with equal acquisition parameters.

Collagen Assay

Collagen content in Hs68 fibroblasts was measured by Sircol collagen assay kit (Biocolor, United Kingdom) according to manufacturer's instructions. Hs68 fibroblasts were seeded at a density of 10^5 cells/ well in 6 well plates. Hs68 fibroblasts were incubated with 20% ADM for 7 days at 37°C in a 5% CO_2 atmosphere and liposomal samples were added at day 3. At day 7, the medium was removed and washed with PBS. Pepsin solution (10 mg/ml in 0.5 M cold acetic acid) was added to each well for collagen isolation. After the supernatants were transferred to other eppendorf tubes, 1 ml Alkali Reagent was added and mixed by inverting contents for 30 min, then the samples were centrifuged at 12,000 × *g* for 10 min at 4°C. Afterward, the supernatant was removed and 750 μl ice-cold Acid-Salt Wash Reagent was added into the collagen-dye pellet to eliminate unbound dye from the surface of the pellet. After centrifuged at 12,000 × g for 10 min, the supernatants were removed and 250 μl Alkali Reagent was added to each sample to dissolve the dye. Finally, the absorbance was measure by an ELISA reader at a wavelength of 555 nm.

Elastin Assay

Elastin content in Hs68 fibroblasts was measured by Fastin elastin assay kit (Biocolor, United Kingdom) according to manufacturer's instructions. Firstly, Hs68 fibroblasts were seeded at a density of 10^5 cells/ well in 6 well plates. Hs68 fibroblasts were incubated with 20% ADM for 7 days at 37°C in a 5% CO_2 atmosphere, and test samples were treated to cells at day 3. At day 7, the medium was removed and washed with PBS. Then, 1 ml of trypsin solution was added into each well to detach Hs68 fibroblasts, and cell lysing solution was transferred to a 1.5 ml micro centrifuge tube. In order to convert cell bound elastin to water soluble α-elastin, 300 μl of cell lysing solution was mixed with 100 μl of 1.0 M oxalic acid to make the final concentration of 0.25 M and heated at 100°C for an

hour. Afterward, an equal volume of elastin precipitating reagent was added and vortexed gently for 15 min to make sure the complete precipitation. After centrifugation at 10,000 g and 4°C for 10 min, the supernatant was discarded and replaced with 1ml of Dye Reagent. The samples were shaken evenly for 90 min and centrifuged at 10,000 *g* for another 10 min. The unbound dye was drained out and 250 μl of Dye Dissociation Reagent was added to each sample to dissolve the dye. Finally, the absorbance was measure by an ELISA reader at a wavelength of 513 nm.

Quantitative Real-Time PCR

Hs68 fibroblasts were cultured in 6 cm dishes at different cell densities for the studies of TNF-α induction and ADM stimulation. After treatment, the total RNA in cells were extracted using Trizol reagent (Protech Technology, Taiwan) in reference to the manufacturer's instructions. Messenger RNA was subsequently reverse transcribed to complementary DNA following the method of TProfessional basic (Biometra, Germany). The obtained cDNA was quantified to 20 ng and the measurement was conducted in StepOnePlus^{TM} Real-Time PCR system with FastStart DNA Mater-PLUS SYBR Green I (Applied Biosystem, United States). The primary sequences were shown in **Table 1**. The efficiency of DNA amplification was performed using the mean cycle threshold (Ct) method, which represent the number of cycles at which the fluorescence surpasses the threshold. ΔCt value in each group was calculated from Ct values of different genes subtracting the Ct value of GAPDH. The resulting relative mRNA expression was showed as fold change ($2^{-\Delta\Delta Ct}$) relative to the expression values in control cells.

Statistical Analysis

Statistical analysis was completed by using commercial software Minitab 16. Data are presented as means ± standard deviations. All *p*-values were calculated by two-tails *t*-test and paired *t*-test. $P < 0.05$ was considered the significant difference.

RESULTS

Characterization of Liposomal Formulations

Physical property is an important parameter in formulation of liposomes as it could affect drug delivery or interaction between drugs and cells. In the present study, physical characteristics of baicalei-loaded liposomes, including particle size, entrapment efficiency, polydispersity index (PDI) and zeta potential, were assessed. **Table 2** illustrates that the particle sizes of empty liposomes, 10 μg/ml baicalein-loaded liposomes (10 μg/ml BC-Lip) and 20 μg/ml baicalein-loaded liposomes (20 μg/ml BC-Lip) are in the range of 135–171 nm. Liposomes encapsulated with baicalein were found with reduced particle size in a dose-dependent manner. Moreover, an increase of baicalein concentration in liposomes led to a decrease in PDI from 0.546 to 0.462. PDI is an important parameter which is used to describe variation of particle size in a population of particles and hence baicalein-loaded liposomes have a relatively narrow size distribution. Moreover, baicalein-loaded liposomes present a decline in entrapment efficiency from 33.65 to 25.40% when drug concentration increase from 30 to 80 μg/ml. The surface charge of baicalein-loaded liposomes was assessed through zeta potential measurement. Since baicalein-loaded liposomes are mainly composed with phosphatidylcholine, an amphoteric phospholipid, these liposomes should exhibit a neutral surface and hence the zeta potential was around zero. Similar values of zeta potential were obtained among these liposomal formulations, suggesting that the presence of baicalein did not alter the electrophoretic mobility of liposomes. The

TABLE 1 | Primer sequences used Real-Time PCR experiment.

Primer	Sequence (Forward) (5′→3′)	Sequence (Reverse) (5′→3′)
GAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT
TNF-α	GACAAGGTGTACGTGAACATCG	CCACACTGTGTCGCCGTAG
IL-1β	AGCTACGAATCTCCGACCAC	CGTTATCCCATGTGTCGAAGAA
IL-6	TGGCTGAAAAAGATGGATGCT	TCTGCACAGCTCTGGCTTGT
IL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTC
MMP-1	CTGGCCACAACTGCCAAATG	CTGTCCCTGAACAGCCCAGTACTT
MMP-3	ATTCCATGGAGCCAGGCTTTC	CATTTGGGTCAAACTCCAACTGTG
COX-2	GCCCTTCACGTTATTGCAGATG	ATATGTTCTCCTGCCTACTGGAA
LPL	GGACTTGGAGATGTGGACCA	TGCTGCTTCTTTTGGCTCTG
FABP4	AAAGTCAAGAGCACCATAACC	TTCAATGCGAACTTCAGTCC

TABLE 2 | Physical parameters of the liposomal formulations after extrusion.

Drug formulation	Particle size (nm)	Entrapment efficiency (%)	PDI	Zeta potential (mV)
Empty liposomes	171.67 ± 8.92	–	0.546	−1.69
Baicalein-loaded liposomes (10 μg/ml)	154.17 ± 2.34	33.65%	0.503	−2.11
Baicalein-loaded liposomes (20 μg/ml)	135.67 ± 3.45	25.40%	0.462	−1.89

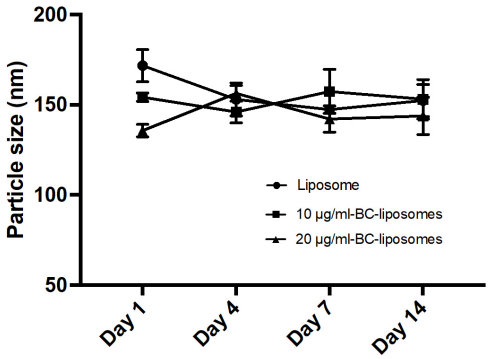

FIGURE 1 | Stability of liposomal formulations. Empty and baicalein (BC)-loaded liposomes were stored at 4°C over different time intervals.

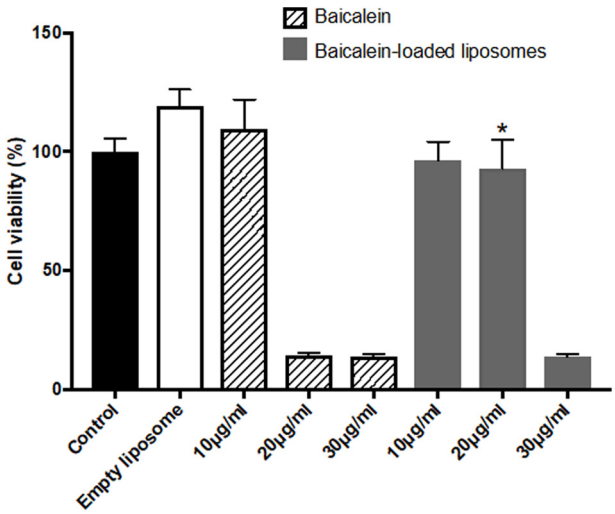

FIGURE 2 | The effect of baicalein, empty and baicalein-loaded liposomes on cell viability of Hs68 fibroblasts. Cells were treated with different concentrations of baicalein and baicalein-loaded liposomes for 24 h and then measured by MTT assay. Cell viability of control was expressed as 100%. The data are presented as means ± standard deviation (n = 4–8). *$p < 0.05$ relative to 20 μg/ml Baicalein-treated cells.

stability of liposomal formulations was further determined through particle size (**Figure 1**). Although particle sizes of empty and baicalein-loaded liposomes were slightly different at day 1, all the liposomes displayed similar stability profiles over a 2-week period at 4°C in the fridge. The stability of liposomes *in vivo* can also be affected by interactions between lipoproteins and few other proteins in the blood. In our previous serum stability studies of liposomes, no significant change in the particle size distribution for all the types of liposomal formulations was noted (Yeh et al., 2015).

Effect of Liposomal Formulations on the Cell Viability, Delivery Efficiency and NO Production

The *in vitro* cytotoxic of free baicalein, empty and baicalein-loaded liposomes on Hs68 fibroblasts and RAW264.7 macrophages were examined using the MTT assay. As shown in **Figure 2**, free baicalein at the concentration of 20 μg/ml was found to reduce cell viability sharply to 14%. In comparison, liposome-encapsulated baicalein at the same concentration showed highly remained cell viability (around 100%), indicating that liposomal formulation was capable to prevent the cytotoxicity of baicalein with significantly enhanced cell viability. However, both free baicalein and liposome-encapsulated baicalein at the concentration of 30 μg/ml showed low cell viability (less than 15%).

Cell uptake of baicalein-loaded liposomes in Hs68 fibroblasts was analyzed based on release of DiI in to cells after cell uptake of DiI-labeled liposomes (Yeh et al., 2015). In here, DAPI stained cell nucleus apparent in blue fluorescence. As shown in **Figure 3A**, Dil-loaded liposomes were gradually taken by cells showing more red fluorescence signals over 24 h. Quantitative analysis of DiI red fluorescence signals demonstrated the fluorescent intensity in cells which were treated with DiI-loaded liposomes was four times higher compared to control cells which were treated with Dil only over 24 h (**Figure 3B**). Hence, liposomal formulation was helpful to improve cell viability and cellular uptake efficiency of baicalein in Hs68 fibroblasts.

To confirm the anti-inflammatory activity of liposome-encapsulated baicalein, cell viability and nitrite production of RAW264.7 were measured. Liposome-encapsulated baicalein showed higher cell viability compared to free drug in treating RAW264.7 macrophages. Baicalein and liposome-encapsulated baicalein dose-dependently reduced cell viability of RAW264.7 macrophages (**Figure 4A**). Based on this result, concentrations with cell viability over 80% were selected for subsequent NO inhibition experiment. The nitrite accumulation in the cells was significantly increased after LPS stimulation. Therefore, cells were simultaneously treated with LPS following by free baicalein, empty or baicalein-loaded liposomes, respectively in order to determine anti-inflammatory activity of baicalein (**Figure 4B**). LPS-induced nitrite production in RAW264.7 macrophages was significantly inhibited through the treatment of free baicalein, empty or baicalein-loaded liposomes. Baicalein inhibited the NO production in a dose-dependent manner, particularly at 20 μg/ml of baicalein (greater than 80% inhibition). liposome-encapsulated baicalein also exhibited the suppressive effect on LPS-induced nitrite production (in approximate 65–75% inhibition), but not in a dose-dependent manner. The result of MTT assay confirmed that baicalein or liposome-encapsulated baicalein had direct anti-inflammatory effects which was not correlated with cell damage (cell viability > 80%).

The Effect of Liposome-Encapsulated Baicalein on Lipid Accumulation and Triglyceride Synthesis in ADM-Induced Hs68 Fibroblasts

Lipogenesis effects of baicalein or liposome-encapsulated baicalein were examined respectively on Hs68 fibroblasts

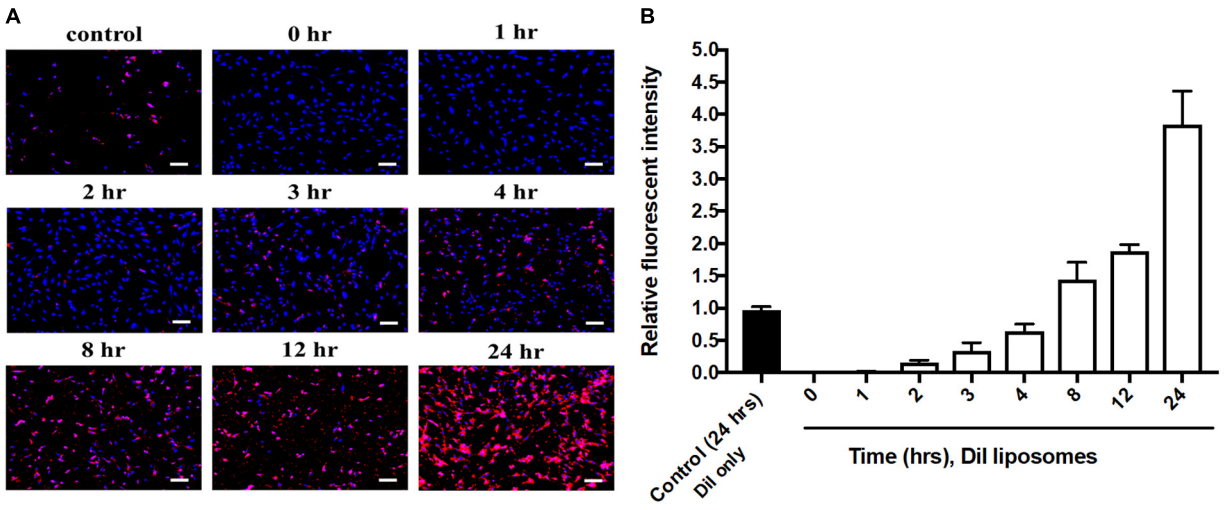

FIGURE 3 | Cellular uptake of DiI-loaded liposomes in Hs68 fibroblasts. **(A)** Hs68 fibroblasts were incubated with DiI and DiI-loaded liposomes at different time intervals (1–24 h). The fluorescent image was photographed under fluorescent microscopy paired with CCD system. **(B)** The fluorescent intensity of DiI-loaded liposomes inside the cells was quantified using Image J. The data are expressed in relative index compared with control. The results are presented as the means ± standard deviation (×100 magnification, scale bar = 200 nm, $n = 4$).

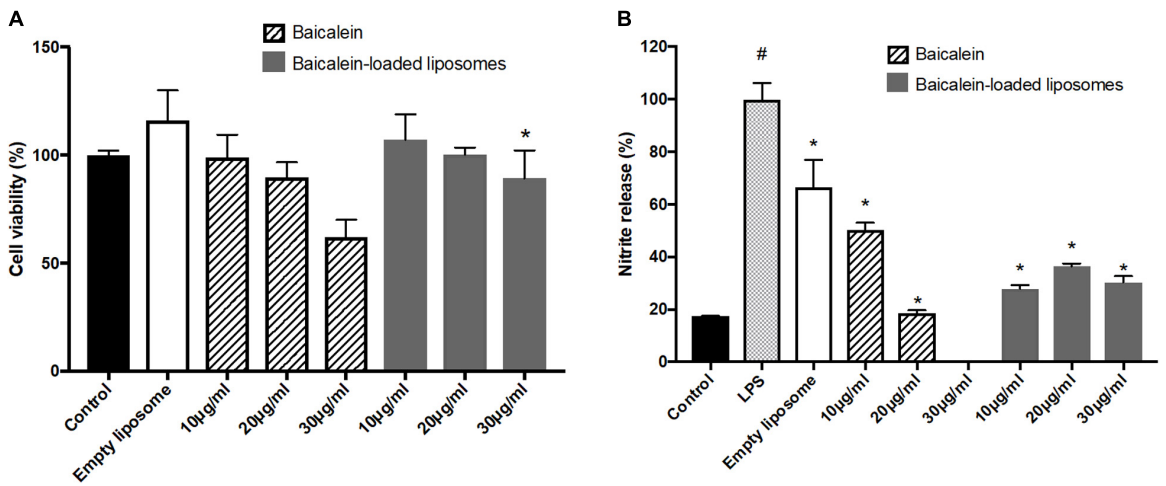

FIGURE 4 | The effect of empty and baicalein-loaded liposomes on cell viability and nitrite production of RAW264.7 macrophages. **(A)** Cells were incubated with baicalein, empty and baicalein-loaded liposomes for 24 h, and then cell viability was measured using MTT assay. $^{*}p < 0.05$ relative to 20 μg/ml Baicalein-treated cells **(B)** RAW264.7 macrophages were induced to inflammation by 500 ng/ml LPS. The inhibitory effect of baicalein and baicalein-loaded liposomes on nitrite production of LPS-induced RAW264.7 macrophages was determined after 24 h of incubation. The total nitrite production in LPS-stimulated cells is expressed as 100%. The data are presented as the means ± standard deviation. $^{\#}P < 0.05$ relative to control and $^{*}p < 0.05$ relative to LPS-stimulated cells ($n = 3$).

within ADM induced lipogenesis for 14 days. Oil Red-O was utilized to measure intracellular lipid accumulation. Under light microscopy, lipid droplets were apparent via treatments of ADM in the presence or absence of empty liposomes (**Figure 5A**). Moreover, cells co-treated with ADM and empty liposomes had a significantly increase in intracellular lipid content compared to the cells treated with ADM only. In contrast, baicalein (10 μg/ ml) showed a significant inhibitory role against ADM-induced lipid formation, while baicalein-loaded liposomes showed comparable inhibitory effects on lipid formation but not dose dependently (**Figure 5B**). These results were further confirmed by quantitative analysis of intracellular triglyceride contents. Suppressive effect on triglyceride accumulation was observed in Hs68 fibroblasts (**Figure 5C**). As expected, ADM induced triglyceride synthesis in Hs68 fibroblasts was significantly suppressed by free baicalein (10 μg/ml), while after liposome-encapsulation, baicalein-loaded liposomes were found to reduce ADM induced triglyceride synthesis, suggesting that a similar but mild effects of baicalein after liposome-encapsulation on the lipid formation and triglyceride synthesis in Hs68 fibroblasts.

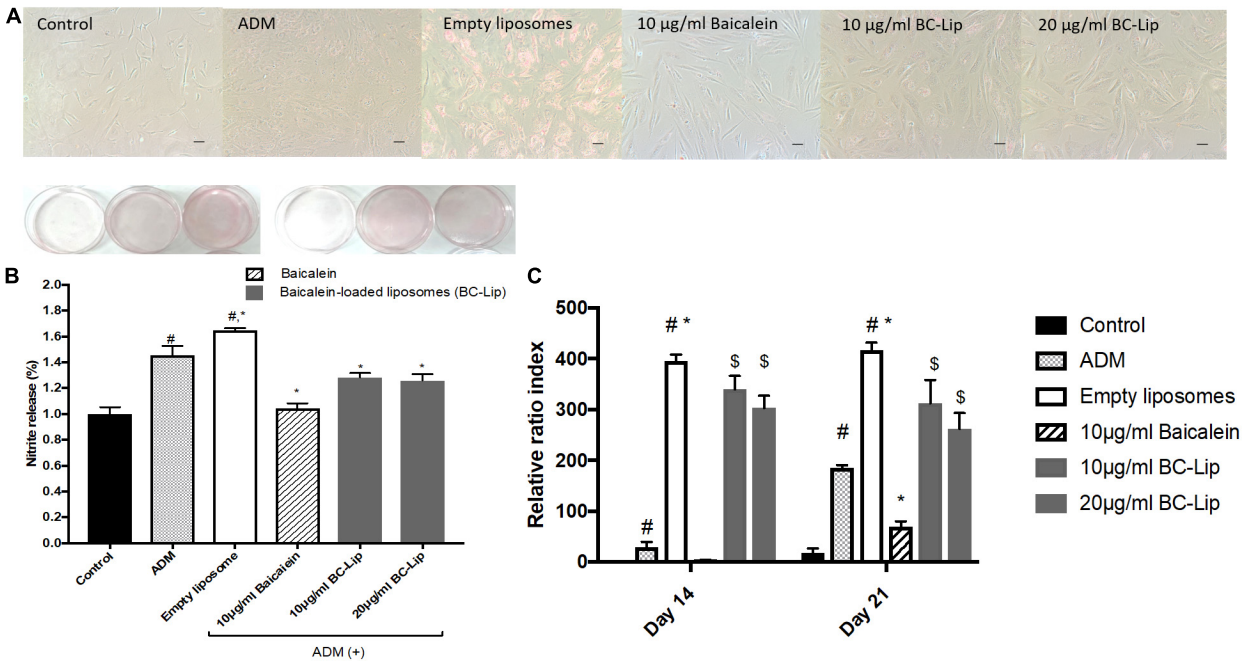

FIGURE 5 | Lipid accumulation in human dermal fibroblasts, Hs68 **(A)** after the differentiation, lipid accumulation in the cells was stained with Oil Red O dye and visualized under a microscope at 100x of magnification. (Scale bar = 200 μm). **(B)** The quantification of lipid accumulation in Hs68 cells was measured using an ELISA reader at 500 nm. The data are presented as relative index to control, and the results were expressed as means ± standard deviation (n = 3–5). **(C)** Intracellular triglyceride content were determined with TG adipogenesis kit at 570 nm in Hs68 cells after 14 and 21 days of incubation with 20% ADM. The results were expressed by the mean intensity of triglyceride compared to control ± standard deviation (n = 3–8). $^{\#}P < 0.05$ relative to control, $^{*}p < 0.05$ relative to cells treated with ADM and $^{\$}p < 0.05$ relative to cells co-treated with ADM and empty liposomes.

The Effect of Liposome-Encapsulated Baicalein on Gene Expressions of Lipogenesis Enzymes in ADM-Induced Hs68 Fibroblasts

To understand the molecular mechanism of baicalein's effects with or not encapsulated in liposomes on lipid accumulation in Hs68 cells, we examined the expression of lipogenesis enzymes, lipoprotein lipase (LPL) and fatty acid binding protein 4 (FABP4). A previous study demonstrated that the gene expressions of LPL and FABP4 in human adipose-derived stromal cells were significantly increased during adipogenic differentiation (D7–D21) and hence they could be served as potential adipogenic differentiation markers (Ambele et al., 2016). Cells treated with ADM, in presence or absence of empty liposomes, resulting in greater gene expression of LPL (2 fold) and FABP4 (sixfold) comparing to control and hence empty liposomes had no additive effect on lipogenesis (**Figure 6**). The increase of these lipogenic enzyme genes indicates that Hs68 fibroblasts can be induced to lipogenesis by ADM treatment no matter the addition of liposomes. In contrast to ADM-induced lipogenesis in Hs68 fibroblasts, free baicalein treatment demonstrated 70 and 48% decrease in LPL and FABP4 expression levels, respectively. Cells co-treated with ADM and liposome-encapsulated baicalein also exhibited suppressive effects on LPL (62% decrease) and FABP4 (49% decrease) mRNA expressions. Such expression profiles of LPL and FABP4 are consistent with lipid and triglyceride levels. Taken together, both free baicalein and liposome-encapsulated baicalein inhibited lipid formation and triglyceride synthesis in Hs68 fibroblasts through suppression of lipogenic enzyme genes.

The Effect of Liposome-Encapsulated Baicalein on Gene Expression of Inflammation-Related Factor After ADM Treatment

As obesity is known to induce mild inflammatory responses, while inflammation plays an important role in insulin resistance, diabetes and other diseases. Hotamisligil reported that the mRNA expression of tumor necrosis factor alpha (TNF-α) mRNA expression in subcutaneous adipose tissue were about 2.5-fold higher in obese women compared to women with normal BMI. Importantly, reduction of body weight (17% w/w) were found to correlate with 22% decrease of TNF-α expression, and lead to indirectly improvement of insulin sensitivity (Hotamisligil et al., 1995). Thus, TNF-α expression was used in the present study as an indicator to determine the release of adipokines in the adipose tissue (Cawthorn and Sethi, 2008). To clarify whether ADM-induced adipogenesis can stimulate inflammation in Hs68 fibroblasts, we measured the mRNA levels of TNF-α. In here, we found that ADM treatment resulted in 1.5-fold increase in the gene expression of TNF-α, and which can be reduced approximate 75 and 80% by baicalein and liposome-encapsulated baicalein respectively (**Figure 7**). In addition, ADM-induced

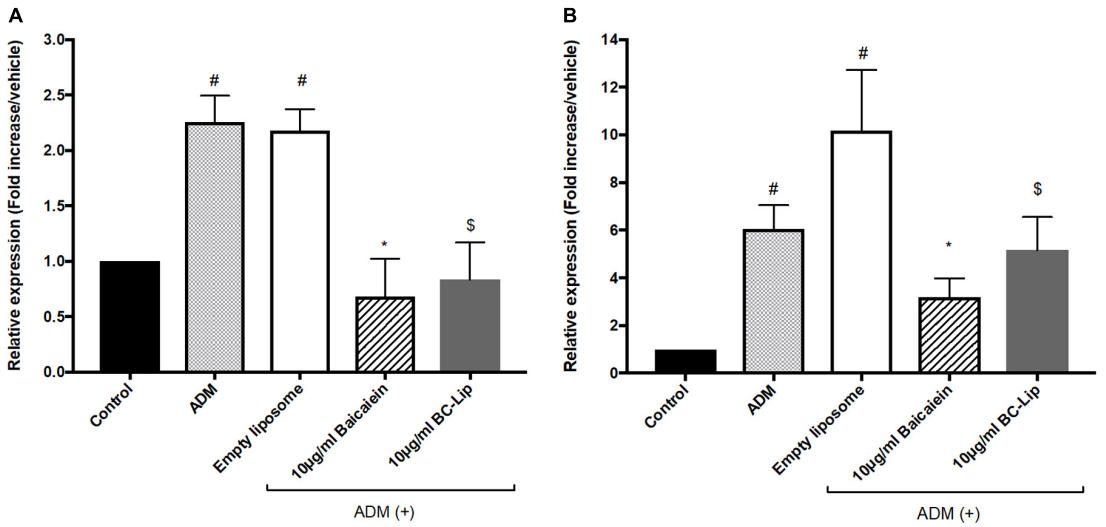

FIGURE 6 | The effect of baicalein, empty and baicalein-loaded liposomes on adipogenic marker genes, LPL **(A)** and FABP4 **(B)** in the presence of ADM. The mRNA expressions of LPL and FABP4 were measured by real-time PCR analysis after 3 days incubation with ADM. Levels of LPL and FABP4 mRNA expression are presented relative to control gene expression. The data were presented as the mean ± standard deviation ($n = 3$). $^{\#}P < 0.05$ relative to control, $^{*}p < 0.05$ relative to cells treated with ADM only and $^{\$}p < 0.05$ relative to cells co-treated with ADM and empty liposomes.

TNF-α expression can be suppressed by liposome-encapsulated baicalein in a dose-dependent manner. A previous report using fresh peritoneal murine macrophages harvested from C57RL/6 mice or ANA-1 macrophage line derived from the bone marrow of C57RL/6 mice demonstrated that LPL can induce TNF-α gene expression and protein secretion (Renier et al., 1994). Moreover, Xu et al. (2015) also indicated that FABP4 could modulate TNF-α secretion in FABP4/aP2 knockout macrophages, suggesting ADM treatment may induce TNF-α expression in Hs68 fibroblasts through the up-regulation of lipogenic enzyme gene expressions (LPL and FABP4).

ADM-induced adipogenesis caused inflammatory responses in Hs68 fibroblasts through the up-regulation of TNF-α gene expression. We further treated Hs68 fibroblasts treated with 20 ng/ml of TNF-α following by measure of the mRNA expressions of inflammatory cytokines, such as cyclooxygenase-2 (COX-2), interleukin-6 (IL-6) and IL-8. As shown in **Figures 8A–C**, incubation of Hs68 fibroblasts with TNF-α increased the gene expressions of COX-2, IL-6 and IL-8 (about 2, 2 and 3.5-fold) and the addition of baicalein, empty and baicalein-loaded liposomes individually down-regulated TNF-α-induced inflammatory gene expressions. Since TNF-α was reported to stimulate ECM degradation by inducing the expression of matrix metalloproteases (MMP)-1 and MMP-3 in dermal fibroblasts (Shindo et al., 2014), we then examined the effect of baicalein, empty and baicalein-loaded liposomes on gene expressions of MMP-1 and MMP-3. Incubation of Hs68 fibroblasts with TNF-α significantly increased gene expressions of MMP-1 and MMP-3 compared to non-treated control cells, whereas baicalein and liposome-encapsulated baicalein significantly reduced TNF-α-induced MMP-1 and MMP-3 mRNA levels (**Figure 8D**). It is interesting to note that empty liposomes have similar inhibitory effect on TNF-α-induced MMP-1 and MMP-3 mRNA expressions but not in statistical significance. Through the up-regulation of TNF-α gene expression, ADM-induced

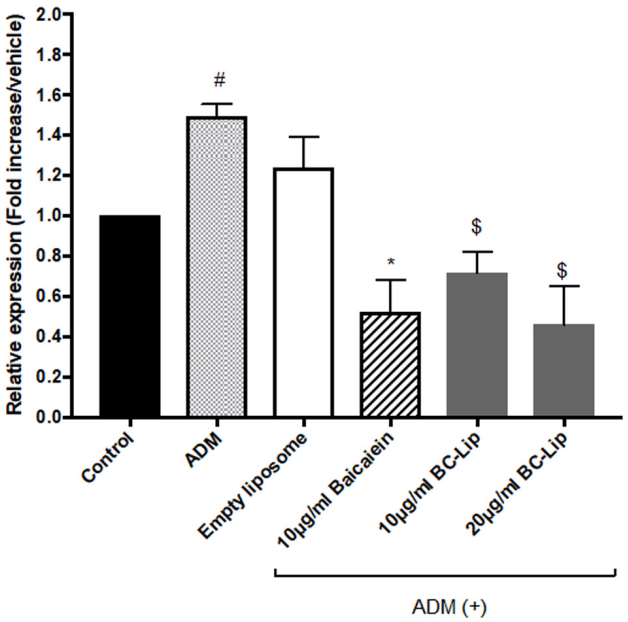

FIGURE 7 | The effect of ADM, Baicalein and baicalein-loaded liposomes on gene expression of TNF-α. The level of TNF-α mRNA expression was measured using RT-PCR. The level of TNF-α mRNA expression is presented relative to control gene expression. The data were presented as the mean ± standard deviation ($n = 3$). $^{\#}P < 0.05$ relative to control, $^{*}p < 0.05$ relative to cells treated with ADM only and $^{\$}p < 0.05$ relative to cells co-treated with ADM and empty liposomes.

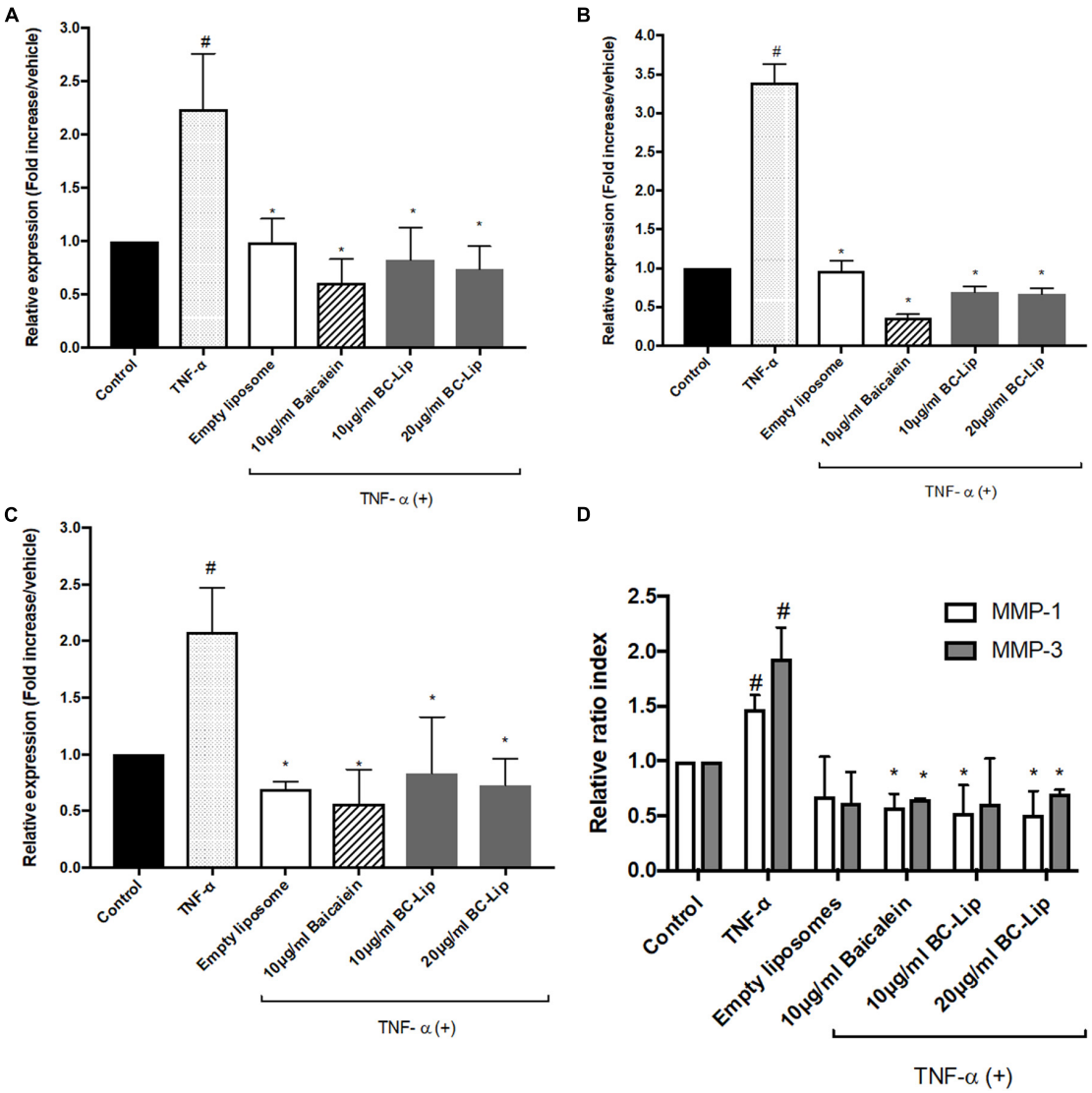

FIGURE 8 | The effect of baicalein, empty and baicalein-loaded liposomes on TNF-α-induced inflammatory responses in Hs68 fibroblasts. Hs68 fibroblasts were treated with test samples in the presence or absence of TNF-α for an hour. Afterward, the gene expressions of COX-2 **(A)**, IL-6 **(B)** and IL-8 **(C)** were determined by real-time PCR. **(D)** The mRNA expressions of MMP-1 and MMP-3 were also measured after 4 h incubation with test samples in the presence or absence of TNF-α. Levels of COX-2, IL-6, IL-8, MMP-1 and MMP-3 mRNA expression are presented relative to control gene expression. The results were expressed as the mean ± standard deviation ($n = 3$). $^{\#}P < 0.05$ relative to control and $^{*}p < 0.05$ relative to cells treated with TNF-α only.

lipogenesis may cause inflammation and ECM degradation in Hs68 fibroblasts. However, baicalein, empty and baicalein-loaded liposomes show their potential to suppress inflammatory responses and MMP expressions via down-regulation of TNF-α pathway.

The Effect of Liposome-Encapsulated Baicalein on ECM Synthesis in ADM-Induced Hs68 Fibroblasts

Dermal fibroblasts have major responsibility to produce ECM for maintaining skin homeostasis and for orchestrating skin tissue regeneration. Since ECM plays a critical role in regulation of skin cell morphogenesis, activity and function, ADM, baicalein and liposome-encapsulated baicalein was further investigated on ECM synthesis. In the presence of ADM and empty liposomes, Hs68 fibroblasts were found enlarged and altered in cell shape. In addition, cells co-treated with ADM and empty (or baicalein-loaded) liposomes produced an elaborate type I collagen matrix (**Figure 9A**), whereas control cells or cells co-treated with ADM and baicalein showed no cross-linked network of collagen microfibers. Quantitative analysis of the fluorescent signals revealed that cells treated by ADM or empty liposomes exhibited higher expression levels of elastin, type I and type III collagens compared to control and cells co-treated with ADM and baicalein (or liposome-encapsulated baicalein) (**Figures 9B–D**).

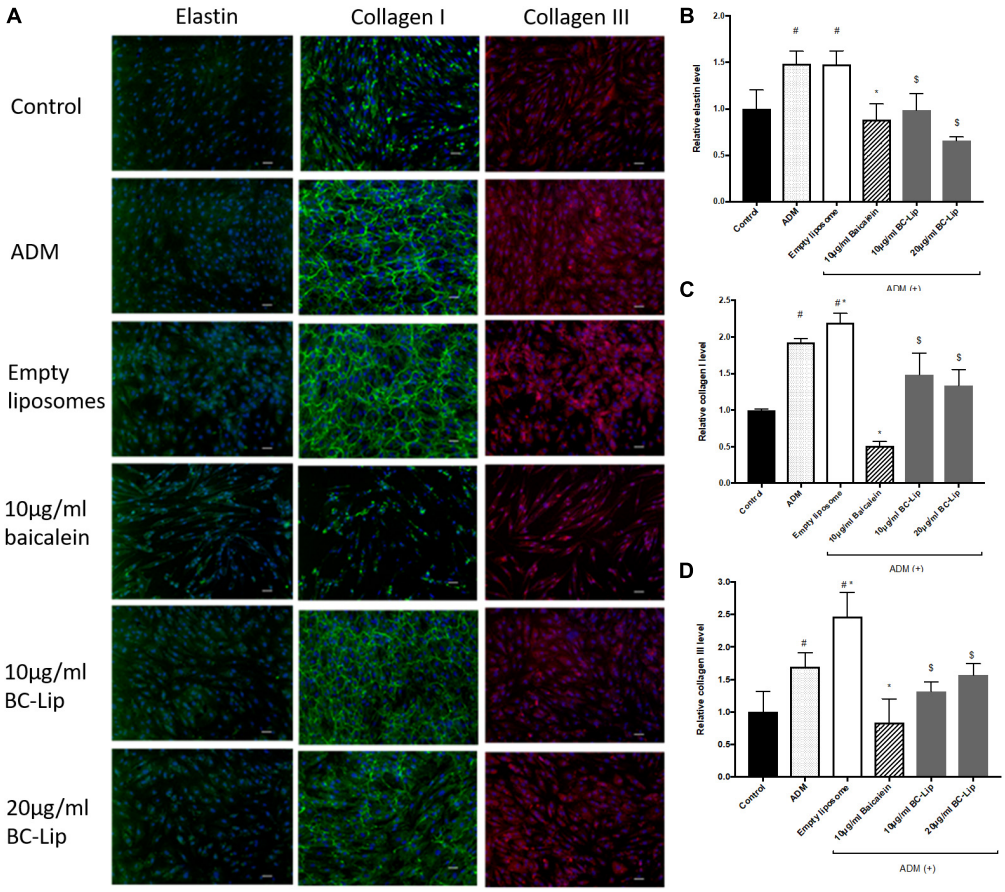

FIGURE 9 | The effect of ADM, baicalein and baicalein-loaded liposomes on elastin, type I and type III collagen protein expressions in Hs68 fibroblasts. Immunofluorescence staining was carried out using specific antibody. **(A)** The immunofluorescent image of elastin, type I and type III collagen were photographed under microscopy paired with CCD system (×100 magnification, Scale bar = 200 μm). Quantification of fluorescent intensity for protein expression levels of **(B)** elastin, **(C)** type I collagen and **(D)** type III collagen was determined by Image J. The levels of protein expression are presented relative to control. The results were expressed as the mean ± standard deviation ($n = 3$). $^{\#}P < 0.05$ relative to control, $^{*}p < 0.05$ relative to cells treated with ADM and $^{\$}p < 0.05$ relative to cells co-treated with ADM and empty liposomes.

These results indicated that baicalein (or liposome-encapsulated baicalein) can largely reduce the stimulatory effect of ADM and liposomal formulation on ECM synthesis.

To further confirm the effect of baicalein and liposome-encapsulated baicalein, collagen and elastin protein level in Hs68 fibroblasts were analyzed using ELISA assay. Results are in agreement with immunohistochemical staining showing a similar declining trend in collagen and elastin production (**Figure 10**). Taken together, ADM and empty liposomes could induce ECM expression level in Hs68 fibroblasts but baicalein or liposome-encapsulated baicalein may have the reverse effects.

DISCUSSION

Previous studies revealed that baicalein is capable of attenuating ROS generation and exhibit high anti-oxidant activity (Chang et al., 2011). We also found that baicalein displays strong DPPH radical scavenging action, particularly at the concentration of 20 μg/ml (around 90%, **Supplementary Figure S1**). In a study reported by Kimura and Sumiyoshi (2011) baicalein could inhibit UV-B-induced MMP-9 and VEGF expression level through suppression of COX-2 but expression of NF-κB and hence baicalein is believed to have a remarkable anti-inflammation activity. In the present study, our findings demonstrate that baicalein is capable to decrease LPS-induced nitrite production in RAW264.7 macrophages and TNF-α stimulated COX-2, IL-6 and IL-8 expressions in Hs68 fibroblasts. These results agree with previous studies, showing that baicalein possesses excellent anti-inflammatory effect (Kimura and Sumiyoshi, 2011; Chen et al., 2014). However, due to its low solubility in aqueous solutions and low bioavailability *in vivo*, medical application of baicalein are very limited, particularly for skin administration. Few recent studies suggested that the therapeutic efficiency of natural compounds could be improved by liposomal nanoencapsulation because of its high compatibility and easy incorporation efficiency (Cadena et al., 2013; Caddeo et al., 2014). Encapsulated drugs in nanoparticles

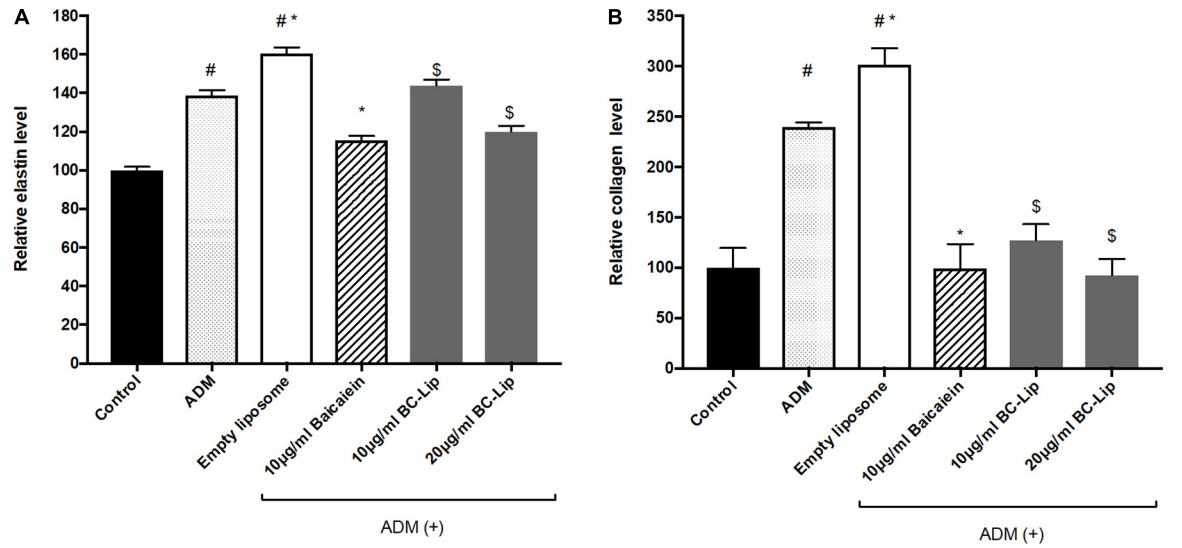

FIGURE 10 | Protein expressions of elastin **(A)** and collagen **(B)** were measured by ELISA. The protein level was presented in relative proportion compared with control. The data were expressed as the mean ± standard deviation ($n = 4$). $^{\#}P < 0.05$ relative to control, $^{*}p < 0.05$ relative to cells treated with ADM only and $^{\$}p < 0.05$ relative to cells co-treated with ADM and empty liposomes.

are known to reduce drugs leakage, prolong the residence time on skin and facilitate the internalization of drugs into cells (Moulaoui et al., 2015). Tsai et al. (2012) found that encapsulated baicalein with nanostructured lipid carrier (NLC) system can be used in brain therapy. In the present study, we used soybean phosphatidylcholine (SPC) for baicalein-loaded liposomal formulations to enhance drug bioavailability. Notably, particle size and PDI were decreased with baicalein–loaded liposomes, and no significant differences in zeta potential was measured between empty liposomes and baicalein-loaded liposomes. The reduction of particle size may be due to stronger drug interactions via hydrogen bonding and hence there is no effect on zeta potential. After 14 days stored at 4°C, only less 20% variation in particle sizes was noted for empty or baicalein-loaded liposomes. Moreover, our previous studies of liposome' stability in serum demonstrated no significant change in the particle size distribution for all the liposomal formulations (Yeh et al., 2015). Hence, liposomal formulations are in high uniformity and homogeneity based on the low PDI and great stability within 14 days. In comparison with free drug, liposome-encapsulated baicalein (20 μg/ml, equal to 74 μM) obtained high cell viability in Hs68 fibroblasts due to the reduction of drug leakage and the anti-inflammatory activity of liposome-encapsulated baicalein is consistent with free drug.

Rakar et al. (2012) showed that primary human dermal fibroblasts were able to differentiate toward adipocytes, osteoblasts and chondrocytes using different induction media. Additionally, Takeda et al. (2017) also observed dramatic morphological change of human dermal fibroblasts over differentiation in which cells morphology convert from characteristic elongated fibroblasts into round adipocyte-like cells. In here, ADM-induced lipogenesis enlarged the cell size of Hs68 fibroblasts with oval and round cells rather than spindle shape. Such morphological changes of Hs68 human dermal fibroblasts during lipogenesis is consistent with the "morphotypes" described in previous studies (Rakar et al., 2012; Takeda et al., 2017). Conversely, Lu et al. (2006) previously demonstrated that baicalein at concentrations of 160–640 μM inhibited the proliferation of porcine preadipocytes with over expression of adipogenesis related genes: PPARγ2 and fatty acid synthase (FAS) but the gene expression was suppressed in lower concentrations of baicalein at 40–320 μM. Similarly, the inhibitory role of baicalein on lipid accumulation in 3T3-L1 fibroblasts and zebrafish was published by Seo et al. (2014). Baicalein inhibited triglyceride accumulation during adipogenesis and significantly down-regulated the gene expression of lipogenesis enzyme, FABP (Cha et al., 2006). In our study, as expected, baicalein or liposome-encapsulated baicalein remained cells with fibroblast-like morphology rather than to adipocytes, and reduced lipid droplet formation in ADM-induced Hs68 fibroblasts. Furthermore, Baicalein and liposome-encapsulated baicalein showed significant inhibitory effect on ADM-induced lipid accumulation and triglyceride synthesis through the suppression of adipogenesis marker, FABP4 and LPL. Thus, we believed that baicalein and liposome-encapsulated baicalein could suppress ADM-induced lipogenesis in human dermal fibroblasts.

As lipogenesis is known to induce inflammatory responses via the increase of TNF-α expression (Hotamisligil et al., 1995; Cawthorn and Sethi, 2008), ADM-induced lipogenesis markedly increased TNF-α expression in Hs68 fibroblasts through the up-regulation of LPL and FABP4 expression, and which was further inhibited by the addition of baicalein, empty and baicalein-loaded liposomes (Renier et al., 1994; Xu et al., 2015). Of note, baicalein, empty liposomes and baicalein-loaded liposomes demonstrated prominent suppression in COX-2, IL-1, IL-6, and IL-8 gene

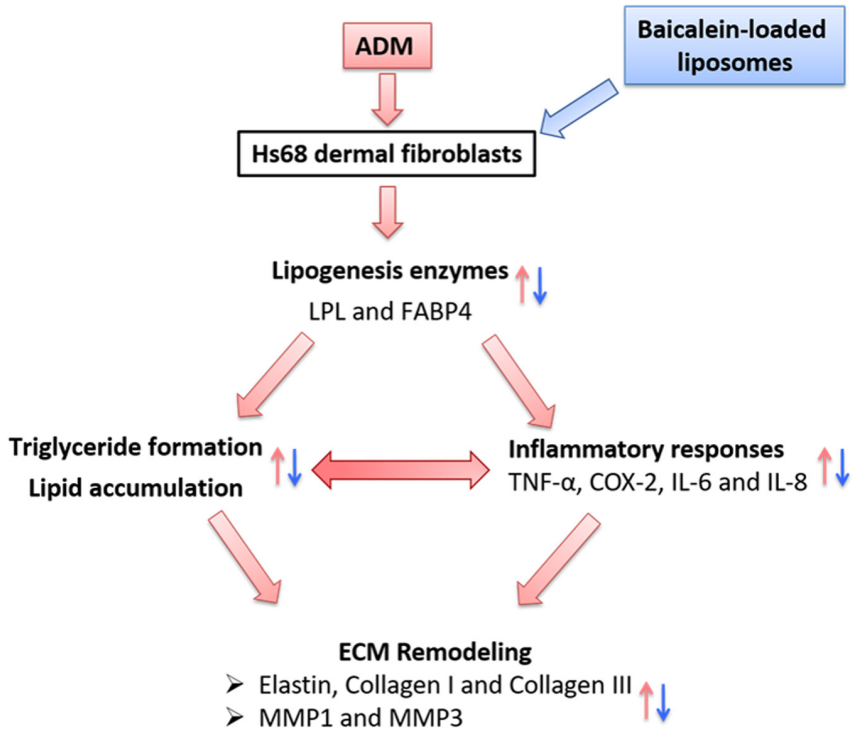

FIGURE 11 | The hypothetic model of how adipogenesis, inflammation and ECM remodeling are regulated in Hs68 human dermal fibroblasts. Physiological stimuli such as ADM may signal through LPL or FABP4 to activate adipogenic and inflammatory pathways, which may be responsible for the upregulation of ECM remodeling.

expressions in TNF-α induced Hs68 fibroblasts. These findings are in agreement with previous studies (Hsieh et al., 2007; Kimura and Sumiyoshi, 2011; Luo et al., 2017), showing baicalein can consistently suppress gene and protein expressions of Cox-2, IL-1, IL-6 and IL-8. Results herein further confirm that liposomes didn't effect on inhibitory role of baicalein on gene expressions of inflammatory responses. Moreover, our results showed that pure baicalein and baicalein-loaded liposomes suppressed gene expression of MMP-1 and MMP-3, while similar results were reported before by Chen et al. (2015) through measuring the gene and protein expressions of MMP-1, 3, and 13 in baicalein treated human OA chondrocytes. They found baicalein can dose-dependently reduce gene and protein expressions of MMP1, 3 and 13 in IL-1β-induced human OA chondrocytes (Chen et al., 2015), while other researchers also demonstrated that baicalein can down-regulate gene and protein expressions of MMP-1 in H_2O_2-treated human HaCaT keratinocytes (Kim et al., 2012). According to our data, baicalein and baicalein-loaded liposomes have similar inhibitory effect on gene expressions of MMP-1 and MMP-3 and that is consistent with previous findings (Kim et al., 2012; Chen et al., 2015). Besides, baicalein inhibited the gene and protein expressions of MMP-2 and MMP-9 and meanwhile promoted the expressions of tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in hepatocellular carcinoma MHCC97H cells and mouse melanoma B16F10 cells (Chen et al., 2013; Choi et al., 2017), suggesting that baicalein can suppress inflammatory responses through the down-regulation of TNF-α expression, resulting in the inhibition of MMP expressions in Hs68 fibroblasts.

Since ECM is playing a key role in skin regeneration, we therefore examined protein expressions of elastin, type I and type III collagens in Hs68 fibroblasts. Our results indicated that ADM stimulation up-regulated elastin, type I and type III collagens in Hs68 fibroblasts. Of note, liposomes slightly increased type I and type III collagens but not elastin as compared with ADM-treated cells. Similar to our findings, human subcutaneous adipose-derived cells were found to have presence of COL1A1 gene (Mariman and Wang, 2010). In a DNA microarray analysis of subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) in Wistar rats, ECM-related genes such as type I, III, and V collagen were expressed higher in SAT than VAT (Mori et al., 2014). Moreover, collagen type I protein was highly expressed and formed a fibrous structure in rat SAT as well as dermis but not in rat VAT. In comparison with undifferentiated cells, a decrease of type I, type III and type V collagens was observed in 3T3-L1 cells during early phase of adipogenic differentiation (Wang et al., 2004; Mori et al., 2014). Enlarged adipocytes reduced 3T3-L1 fibroblast proliferation and gene expression of collagen type I and elastin and increased gene expression of MMP 13 (Ezure and Amano, 2011). Therefore, Hs68 fibroblast may present similar ECM expressions (such as type I and type III collagens) to human subcutaneous adipose-derived cells, rat SAT and VAT but not mouse 3T3-L1 adipocytes. Besides, baicalein and liposome-encapsulated baicalein suppressed the

protein expressions of elastin, type I and type III collagen in ADM-induced Hs68 fibroblasts which may due to the inhibition of lipogenesis. Therefore, we elucidated that lipogenesis in Hs68 fibroblasts can increase ECM formulation, particularly in the protein expression of elastin, type I and III collagens. It is worth noting that baicalein had inhibitory effect on ECM formation in lipid-containing fibroblasts.

In summary, we propose the following network: Physiological stimuli (ADM treatment) influenced the expressions of lipogenic enzyme genes (LPL and FABP4); simultaneously, lipogenesis enzymes control the lipid accumulation and inflammatory responses, which are the key factors for ECM remodeling in Hs68 fibroblasts (**Figure 11**). Besides, Liposome-encapsulated baicalein provide enhanced cell viability and cellular uptake efficiency of Hs68 fibroblasts together with down-regulation of ADM-induced lipid accumulation and ECM formation in Hs68 fibroblasts through suppression of lipogenesis enzymes and inflammatory responses. In conclusion, we suggested that liposome-encapsulated baicalein can provide an opportunity as medical or cosmetic products to prevent lipogenesis and maintain ECM structure in skin.

AUTHOR CONTRIBUTIONS

C-LF designed and performed the experiments and analyzed the data. KT performed the experiments. YW wrote the manuscript and contributed to data analysis. H-IC supervised the project, contributed to data analysis, and wrote the manuscript.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00155/full#supplementary-material

FIGURE S1 | The anti-oxidant activity of baicalein measured by DPPH scavenging assay.

REFERENCES

Ambele, M. A., Dessels, C., Durandt, C., and Pepper, M. S. (2016). Genome-wide analysis of gene expression during adipogenesis in human adipose-derived stromal cells reveals novel patterns of gene expression during adipocyte differentiation. *Stem Cell Res.* 16, 725–734. doi: 10.1016/j.scr.2016.04.011

Bergstrand, N., Arfvidsson, M. C., Kim, J. M., Thompson, D. H., and Edwards, K. (2003). Interactions between pH-sensitive liposomes and model membranes. *Biophys. Chem.* 104, 361–79. doi: 10.1016/S0301-4622(03)00011-5

Bhattacharya, M. (2016). Mesenchymal metamorphosis. *Sci. Transl. Med.* 8:370ec202. doi: 10.1126/scitranslmed.aal3700

Caddeo, C., Díez-Sales, O., Pons, R., Fernàndez-Busquets, X., Fadda, A. M., and Manconi, M. (2014). Topical anti-inflammatory potential of quercetin in lipid-based nanosystems: in vivo and in vitro evaluation. *Pharm. Res.* 31, 959–968. doi: 10.1007/s11095-013-1215-1210

Cadena, P. G., Pereira, M. A., Cordeiro, R. B., Cavalcanti, I. M., Neto, B. B., Maria do Carmo, C. B., et al. (2013). Nanoencapsulation of quercetin and resveratrol into elastic liposomes. *Biochimica et Biophysica Acta* 1828, 309–316. doi: 10.1016/j.bbamem.2012.10.022

Cawthorn, W. P., and Sethi, J. K. (2008). TNF-alpha and adipocyte biology. *FEBS Lett.* 582, 117–131. doi: 10.1016/j.febslet.2007.11.051

Cha, M. H., Kim, I. C., Lee, B. H., and Yoon, Y. (2006). Baicalein inhibits adipocyte differentiation by enhancing COX-2 expression. *J. Med. Food* 9, 145–153. doi: 10.1089/jmf.2006.9.145

Chang, W. T., Li, J., Haung, H. H., Liu, H., Han, M., Ramachandran, S., et al. (2011). Baicalein protects against doxorubicin-induced cardiotoxicity by attenuation of mitochondrial oxidant injury and JNK activation. *J. Cell. Biochem.* 112, 2873–2881. doi: 10.1002/jcb.23201

Chen, K., Zhang, S., Ji, Y., Li, J., An, P., Ren, H., et al. (2013). Baicalein inhibits the invasion and metastatic capabilities of hepatocellular carcinoma cells via down-regulation of the ERK pathway. *PLoS One* 8:e72927. doi: 10.1371/journal.pone.0072927

Chen, S., Yang, Y., Feng, H., Wang, H., Zhao, R., and Liu, H. (2014). Baicalein inhibits interleukin-1β-induced proliferation of human rheumatoid arthritis fibroblast-like synoviocytes. *Inflammation* 37, 163–169. doi: 10.1007/s10753-013-9725-9729

Chen, W. P., Xiong, Y., Hu, P. F., Bao, J. P., and Wu, L. D. (2015). Baicalein inhibits MMPs expression via a MAPK-dependent mechanism in chondrocytes. *Cell Physiol. Biochem.* 36, 325–333. doi: 10.1159/000374075

Choi, E. O., Cho, E. J., Jeong, J. W., Park, C., Hong, S. H., Hwang, H. J., et al. (2017). Baicalein inhibits the migration and invasion of B16F10 mouse melanoma cells through inactivation of the PI3K/Akt signaling pathway. *Biomol. Ther.* 25, 213–221. doi: 10.4062/biomolther.2016.094

de Oliveira, M. R., Nabavi, S. F., Habtemariam, S., Erdogan Orhan, I., Daglia, M., and Nabavi, S. M. (2015). The effects of baicalein and baicalin on mitochondrial function and dynamics: a review. *Pharmacol. Res.* 100, 296–308. doi: 10.1016/j.phrs.2015.08.021

Ezure, T., and Amano, S. (2010). Increased subcutaneous adipose tissue impairs dermal function in diet-induced obese mice. *Exp. Dermatol.* 19, 878–882. doi: 10.1111/j.1600-0625.2009.00970.x

Ezure, T., and Amano, S. (2011). Negative regulation of dermal fibroblasts by enlarged adipocytes through release of free fatty acids. *J. Invest. Dermatol.* 131, 2004–2009. doi: 10.1038/jid.2011.145

Fang, C. L., Huang, L. H., Tsai, H. Y., and Chang, H. I. (2016). Dermal lipogenesis inhibits adiponectin production in human dermal fibroblasts while exogenous adiponectin administration prevents against UVA-induced dermal matrix degradation in human skin. *Int. J. Mol. Sci.* 17:E1129. doi: 10.3390/ijms17071129

Galović Rengel, R., Barisić, K., Pavelić, Z., Zanić Grubisić, T., Cepelak, I., and Filipović-Grcić, J. (2002). High efficiency entrapment of superoxide dismutase into mucoadhesive chitosan-coated liposomes. *Eur. J. Pharm. Sci.* 15, 441–448. doi: 10.1016/S0928-0987(02)00030-1

Gao, Y., Lu, J., Zhang, Y., Chen, Y., Gu, Z., and Jiang, X. (2013). Baicalein attenuates bleomycin-induced pulmonary fibrosis in rats through inhibition of miR-21. *Pulm. Pharmacol. Ther.* 26, 649–654. doi: 10.1016/j.pupt.2013.03.006

Hew, J., Solon-Biet, S. M., McMahon, A. C., Ruohonen, K., Raubenheimer, D., Ballard, J. W., et al. (2016). The effects of dietary macronutrient balance on skin structure in aging male and female mice. *PLoS One* 11:e0166175. doi: 10.1371/journal.pone.0166175

Hotamisligil, G. S., Arner, P., Caro, J. F., Atkinson, R. L., and Spiegelman, B. M. (1995). Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J. Clin. Invest.* 95, 2409–2415. doi: 10.1172/JCI117936

Hsieh, C. J., Hall, K., Ha, T., Li, C., Krishnaswamy, G., and Chi, D. S. (2007). Baicalein inhibits IL-1beta- and TNF-alpha-induced inflammatory cytokine production from human mast cells via regulation of the NF-kappaB pathway. *Clin. Mol. Allergy* 5:5.

Huang, Y., Zhang, B., Gao, Y., Zhang, J., and Shi, L. (2014). Baicalein-nicotinamide cocrystal with enhanced solubility, dissolution, and oral bioavailability. *J. Pharm. Sci.* 103, 2330–2337. doi: 10.1002/jps.24048

Kalepu, S., and Nekkanti, V. (2015). Insoluble drug delivery strategies: review of recent advances and business prospects. *Acta Pharm. Sin. B* 5, 442–453. doi: 10.1016/j.apsb.2015.07.003

Kim, K. C., Kang, S. S., Lee, J., Park, D., and Hyun, J. W. (2012). Baicalein attenuates oxidative stress-induced expression of matrix metalloproteinase-1 by regulating the ERK/JNK/AP-1 pathway in human keratinocytes. *Biomol. Ther.* 20, 57–61. doi: 10.4062/biomolther.2012.20.1.057

Kimura, Y., and Sumiyoshi, M. (2011). Effects of baicalein and wogonin isolated from *Scutellaria baicalensis* roots on skin damage in acute UVB-irradiated hairless mice. *Eur. J. Pharmacol.* 661, 124–132. doi: 10.1016/j.ejphar.2011.04.033

Koumas, L., Smith, T. J., Feldon, S., Blumberg, N., and Phipps, R. P. (2003). Thy-1 expression in human fibroblast subsets defines myofibroblastic or lipofibroblastic phenotypes. *Am. J. Pathol.* 163, 1291–1300. doi: 10.1016/S0002-9440(10)63488-8

Li, X., Luo, W., Ng, T. W., Leung, P. C., Zhang, C., Leung, K. C., et al. (2017). Nanoparticle-encapsulated baicalein markedly modulates pro-inflammatory response in gingival epithelial cells. *Nanoscale* 9, 12897–12907. doi: 10.1039/c7nr02546g

Li, Y., Zhang, C., Liu, L., Gong, Y., Xie, Y., and Cao, Y. (2017). The effects of baicalein or baicalin on the colloidal stability of ZnO nanoparticles (NPs) and toxicity of NPs to Caco-2 cells. *Toxicol. Mech. Methods* 28, 167–176. doi: 10.1080/15376516.2017.1376023

Lu, R. H., Li, Y., Zhang, L. J., and Yang, G. S. (2006). Effects of baicalein on the proliferation and differentiation of pig preadipocyte. *Chin. J. Biotechnol.* 22, 1002–1006. doi: 10.1016/S1872-2075(06)60069-1

Luo, X., Yu, Z., Deng, C., Zhang, J., Ren, G., Sun, A., et al. (2017). Baicalein ameliorates TNBS-induced colitis by suppressing TLR4/MyD88 signaling cascade and NLRP3 inflammasome activation in mice. *Sci. Rep.* 7:16374. doi: 10.1038/s41598-017-12562-6

Mariman, E. C., and Wang, P. (2010). Adipocyte extracellular matrix composition, dynamics and role in obesity. *Cell Mol. Life Sci.* 67, 1277–1292. doi: 10.1007/s00018-010-0263-4

Mohammed, A. R., Weston, N., Coombes, A. G., Fitzgerald, M., and Perrie, Y. (2004). Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. *Int. J. Pharm.* 285, 23–34. doi: 10.1016/j.ijpharm.2004.07.010

Mori, S., Kiuchi, S., Ouchi, A., Hase, T., and Murase, T. (2014). Characteristic expression of extracellular matrix in subcutaneous adipose tissue development and adipogenesis; comparison with visceral adipose tissue. *Int. J. Biol. Sci.* 10, 825–833. doi: 10.7150/ijbs.8672

Moulaoui, K., Caddeo, C., Manca, M. L., Castangia, I., Valenti, D., Escribano, E., et al. (2015). Identification and nanoentrapment of polyphenolic phytocomplex from *Fraxinus angustifolia*: in vitro and in vivo wound healing potential. *Eur. J. Med. Chem.* 89, 179–188. doi: 10.1016/j.ejmech.2014.10.047

Ong, S. G., Ming, L. C., Lee, K. S., and Yuen, K. H. (2016). Influence of the encapsulation efficiency and size of liposome on the oral bioavailability of griseofulvin-loaded liposomes. *Pharmaceutics* 8:E25. doi: 10.3390/pharmaceutics8030025

Rajkumari, J., Busi, S., Vasu, A. C., and Reddy, P. (2017). Facile green synthesis of baicalein fabricated gold nanoparticles and their antibiofilm activity against *Pseudomonas aeruginosa* PAO1. *Microb. Pathog.* 107, 261–269. doi: 10.1016/j.micpath.2017.03.044

Rakar, J., Lönnqvist, S., Sommar, P., Junker, J., and Kratz, G. (2012). Interpreted gene expression of human dermal fibroblasts after adipo-, chondro- and osteogenic phenotype shifts. *Differentiation* 84, 305–313. doi: 10.1016/j.diff.2012.08.003

Rehan, V. K., Sugano, S., Wang, Y., Santos, J., Romero, S., Dasgupta, C., et al. (2006). Evidence for the presence of lipofibroblasts in human lung. *Exp. Lung Res.* 32, 379–393. doi: 10.1080/01902140600880257

Renier, G., Skamene, E., DeSanctis, J. B., and Radzioch, D. (1994). Induction of tumor necrosis factor alpha gene expression by lipoprotein lipase. *J. Lipid Res.* 35, 271–278.

Schmidt, B. A., and Horsley, V. (2013). Intradermal adipocytes mediate fibroblast recruitment during skin wound healing. *Development* 140, 1517–1527. doi: 10.1242/dev.087593

Seo, M. J., Choi, H. S., Jeon, H. J., Woo, M. S., and Lee, B. Y. (2014). Baicalein inhibits lipid accumulation by regulating early adipogenesis and m-TOR signaling. *Food Chem. Toxicol.* 67, 57–64. doi: 10.1016/j.fct.2014.02.009

Shindo, S., Hosokawa, Y., Hosokawa, I., Ozaki, K., and Matsuo, T. (2014). Genipin inhibits MMP-1 and MMP-3 release from TNF-a-stimulated human periodontal ligament cells. *Biochimie* 107(Pt B), 391–395. doi: 10.1016/j.biochi.2014.10.008

Takeda, Y., Harada, Y., Yoshikawa, T., and Dai, P. (2017). Direct conversion of human fibroblasts to brown adipocytes by small chemical compounds. *Sci. Rep.* 7:4304. doi: 10.1038/s41598-017-04665-x

Tsai, M. J., Wu, P. C., Huang, Y. B., Chang, J. S., Lin, C. L., Tsai, Y. H., et al. (2012). Baicalein loaded in tocol nanostructured lipid carriers (tocol NLCs) for enhanced stability and brain targeting. *Int. J. Pharm.* 423, 461–470. doi: 10.1016/j.ijpharm.2011.12.009

Wang, P., Mariman, E., Keijer, J., Bouwman, F., Noben, J. P., Robben, J., et al. (2004). Profiling of the secreted proteins during 3T3-L1 adipocyte differentiation leads to the identification of novel adipokines. *Cell Mol. Life Sci.* 61, 2405–2417. doi: 10.1007/s00018-004-4256-z

Wojciechowicz, K., Gledhill, K., Ambler, C. A., Manning, C. B., and Jahoda, C. A. (2013). Development of the mouse dermal adipose layer occurs independently of subcutaneous adipose tissue and is marked by restricted early expression of FABP4. *PLoS One* 8:e59811. doi: 10.1371/journal.pone.0059811

Xu, H., Hertzel, A. V., Steen, K. A., Wang, Q., Suttles, J., and Bernlohr, D. A. (2015). Uncoupling lipid metabolism from inflammation through fatty acid binding protein-dependent expression of UCP2. *Mol. Cell. Biol.* 35, 1055–1065. doi: 10.1128/MCB.01122-14

Yeh, C. C., Su, Y. H., Lin, Y. J., Chen, P. J., Shi, C. S., Chen, C. N., et al. (2015). Evaluation of the protective effects of curcuminoid (curcumin and bisdemethoxycurcumin)-loaded liposomes against bone turnover in a cell-based model of osteoarthritis. *Drug Des. Devel. Ther.* 9, 2285–2300. doi: 10.2147/DDDT.S78277

Zeng, C., Jiang, W., Tan, M., Yang, X., He, C., Huang, W., et al. (2016). Optimization of the process variables of tilianin-loaded composite phospholipid liposomes based on response surface-central composite design and pharmacokinetic study. *Eur. J. Pharm. Sci.* 85, 123–131. doi: 10.1016/j.ejps.2016.02.007

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The Pathways for Layered Double Hydroxide Nanoparticles to Enhance Antigen (Cross)-Presentation on Immune Cells as Adjuvants for Protein Vaccines

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Nanoparticles (NPs) are intensively investigated as adjuvants in new generation vaccines, while how these NPs promote the immune responses has not been well understood. In this research, we have tried to elucidate the possible pathways for layered double hydroxide (LDH) NPs to provoke immune responses. As previously reported, LDH NPs efficiently deliver antigens to antigen presenting cells (APCs). In this research, we have found that these internalized LDH NPs are not released by these APCs within 8 h. We have for the first time found that macrophage cells exchange the internalized LDH NPs with other surrounding ones, which may promote immune responses in an additional way. Moreover, the internalized LDH-antigen NPs significantly facilitate the maturation of immature DCs and enhance cross-presentation of epitope/MHC class I complexes on the DC surface. This research would help understand the NP adjuvant mechanism and further assist the design of new specific NPs as more efficient nano-adjuvants.

Keywords: nanoparticle adjuvant, layered double hydroxides, antigen presentation cells, dendritic cell maturation, cross-presentation, immune responses

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INTRODUCTION

Various nanomaterials have been widely examined to deliver immunogens and immune stimulants as adjuvants in vaccine development (Fan and Moon, 2015; Zhang et al., 2015). For example, polystyrene delivering ovalbumin (OVA) induces systemic immune responses in sheep (Scheerlinck et al., 2006). Poly (lactic-co-glycolic acid) (PLGA) co-delivering tyrosinase-related protein 2 (TRP-2) and toll-like receptor ligand 4 (TLR4) induces cell-mediated immunity (Hamdy et al., 2008). Mesoporous silica (MS) as the antigen adjuvant also shows good adjuvant activities in HIV (Cheng et al., 2012) and porcine circovirus (Guo et al., 2012) vaccines. Calcium phosphate (Cap) adjuvanted herpes simplex vaccine shows systemic immune response in mice (He et al., 2002) and layered double hydroxide (LDH) delivering DNA vaccine shows high efficiency in transfection, and promotes immunity (Li et al., 2011; Wang et al., 2014; Williams et al., 2014). In particular, we have previously reported that LDH co-delivering OVA/TLR9 ligand CpG and Intimin β (IB) promotes potent humoral and cell-mediated immunities (Yan et al., 2014, 2018; Chen et al., 2016). However, how nanomaterials as adjuvants stimulate strong immune responses has not been well understood.

The most critical adjuvant processes include the assisted cellular uptake of antigen and subsequent antigen presentation or cross-presentation by antigen-presenting cells (APCs) (Toes et al., 1996). After subcutaneous administration of vaccine formulations, APCs are recruited to take up the nanomaterial-antigen particles, and then circulate to the local regional nodes. During this period, the nanomaterial-antigen particles are processed within the APCs to present the epitope and prime naïve lymphocytes (Slingluff, 2011). Therefore, the understanding of APCs' cellular uptake and APCs' antigen (cross)-presentation pathways via nanoparticles is very important in adjuvant design and development.

It has been confirmed that LDH NPs facilitate negatively charged antigens (such as BSA) to attach onto and enter the cell (Gu et al., 2015; Chen et al., 2016). As reported by Li et al. (2010) 60–65% bone marrow dendritic cells (BMDCs) took up LDH nanoparticles within 3 h. LDH is a family of anionic clay minerals, with the general formula of $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]^{x+}[A^{n-}_{x/n}\cdot yH_2O]^{x-}$, where M^{2+} is a divalent cation, M^{3+} a trivalent cation, and A^{n-} an anion (Braterman et al., 2004). LDH has positively charged hydroxide basal layers where the trivalent cations substitute for the divalent cations, which are balanced by the hydrated anions intercalated in the interlayer space. MgAl-LDH NPs possess low toxicity and good biocompatibility, high loading of proteins and proteomic vaccines and a high capability to facilitate the cellular uptake of payloads (Xu et al., 2006c, 2008a), which may explain why LDH NPs can act as effective adjuvants to stimulate strong immune responses in vaccine development (Yan et al., 2014). However, there is no report regarding (1) whether LDH-antigen complexes facilitate maturation of APCs; (2) whether APCs that take up NPs exchange these NPs with other APCs; and (3) how antigen is cross-presented by APCs through LDH-antigen complexes.

In this study, we reinvestigated the antigen cellular uptake of LDH-dye NPs by murine macrophage cells and bone-marrow dendritic cells, and examined enhancement of presentation and cross-presentation of the model antigen OVA delivered by LDH NPs. We also employed the mimicking of antigen presentation via MHC class I pathway using LDH NPs to prime T cell activation in B3Z CD8+ T hybridoma system. Our results demonstrate the possible pathways to explain how LDH-delivered antigen significantly improves the dendritic cells maturation and enhances the antigen cross-presentation on DCs' surface.

MATERIALS AND METHODS

Preparation of LDH, LDH-FITC, and LDH-Congo Red (LDH-CR) NPs

$Mg_2Al(OH)_6Cl\cdot mH_2O$ LDH NPs were prepared as described in previous work (Xu et al., 2006a,b). In brief, 10 mL of mixed salt solution containing $MgCl_2$ $6H_2O$, (0.30 M) (Chem-Supply, 99.0–101.0%), with $AlCl_3$ $6H_2O$ (0.10 M) (Scharlau, 95–101%) was poured into 40 mL of NaOH (Sodium hydroxide pellets; Ajax Finechem) solution (0.15 M) under vigorous stirring. After 10 min stirring, LDH slurry was collected and washed twice with deionized water by centrifugation (SIGMA4®-16K Centrifuge) at 4700 rpm for 10 min. Then the slurry was manually dispersed in 40 mL of deionized water and transferred into an stainless steel autoclave with a Teflon lining (Parr Acid Digestion Vessels) for heating at 100°C for 6 h, giving rise to a homogeneously dispersed MgAl-LDH suspension.

To make LDH-FITC NPs, ¼ of manually dispersed LDH slurry was mixed with 0.5 mL of 0.025 M $FITC^{2-}$ (fluorescein isothiocyanate; Sigma-Aldrich)„ and shaken for 1 h, followed by separation and washing via centrifugation. The slurry was then manually dispersed in 10 mL of deionized water, which was similarly treated at 100°C for 6 h, yielding a well dispersed LDH-FITC NP suspension.

LDH-Congo red (LDH-CR) NPs were prepared similarly. Congo-red (0.0125 M; Sigma-Aldrich) was pre-mixed with 40 mL NaOH (0.15 M) solution before adding 10 mL of mixed salt solution containing $MgCl_2$ (0.30 M) and $AlCl_3$ (0.10 M). The resultant suspension was separated and the collected slurry washed twice. Finally, the slurry was dispersed in water and treated in an autoclave at 100°C for 14 h, yielding an LDH-CR NP suspension.

The particle size distribution of these LDH NP suspensions was measured with a dynamic light scattering (DLS) instrument (Nanosizer Nano ZS, MALVERN Instruments) to estimate the average hydrodynamic particle size and check the dispersion state.

Cell Culture

RAW 264.7 macrophage cells (ATCC) were grown on 93 mm × 21 mm Petri dish in complete RPMI 1640 medium (Life Technologies Corporation, Australia) supplemented with 10% fetal bovine serum and adjusted to contain 100 μg/mL streptomycin and 100 units/mL penicillin, all from Invitrogen. Cell subcultures were made by scraping or mechanical isolation.

DC2.4 cells (kindly provided by A/Prof Mingnan Chen, University of Utah, United States) were grown in complete RPMI 1640 medium supplemented with 10% fetal bovine serum and adjusted to contain 1% L-glutamine, streptomycin and penicillin, all from Invitrogen.

Bone marrow dendritic cells were generated according a previous publication (Lutz et al., 1999). All animal studies were performed with adherence to the guidelines of the Animal Ethics Committee of The University of Queensland. Femurs and tibias were obtained from 6 to 8 weeks C57BL/6 female mice. Bone marrow was mashed into the single cell suspension via a 70 μm cell strainer. On the first day, 2×10^6 BM leukocytes were seeded in each Petri dish in 10 mL complete RPMI medium with 10% fetal bovine serum and 0.05 mM of 2-mercaptoethanol and adjusted to contain 1% L-glutamine, streptomycin, and penicillin. In addition, 200 ng recombinant murine granulocyte macrophage colony stimulating factor (rmGM-CSF; Sigma-Aldrich) was added as supplement. At day 3, another 200 ng rmGM-CSF in 10 mL medium was added into the dish. At days 6 and 8, half of cell supernatant was collected, and after centrifugation, cells were resuspended into 10 mL fresh medium with 200 ng rmGM-CSF, and then added into original Petri dish.

Cellular Uptake of LDH NPs

After subculture of RAW 264.7 macrophage cells, 35 mm × 10 mm Nunclon cell culture dishes were used for cell growth at the density of 5×10^5 cells/mL for overnight. Then, 5 or 25 μg/mL LDH-FITC NP suspension was added into these dishes for cellular uptake. Controls were added with the same volume of PBS. Cells were cultured at 37°C in a 5% CO_2 incubator and then collected at the time point of 0.5, 1, 2, 4, or 8 h. The collected cells were washed, and fixed in 4% Paraformaldehyde (PFA; Sigma-Aldrich) solution for FACS analysis (FCM, BD Accuri™ C6, BD Biosciences, San Jose, CA, United States).

Similarly, freshly obtained BMDCs were cultured in 6-well plates at the density of 1×10^6 DC/well in 1.5 mL medium containing 50 μg/mL LDH-FITC NPs. After incubation for 0.5, 1, 2, 4, or 8 h at 37°C, BMDCs were collected for FACS analysis to determine the uptake kinetics. For dose-dependent uptake assay, BMDCs were cultured in 1.5 mL medium containing 10, 20, 50, 100, and 200 μg/mL of LDH-FITC NPs for 2 h at 37°C, and then collected for uptake quantification using FACS. Similarly, the cellular uptake kinetics of LDH-CR NPs were also examined.

Cellular Exocytosis of Internalized LDH NPs

To analyze the release of internalized LDH-FITC NPs by the cells, 25 μg/ml LDH-FITC NP suspension was added into the dishes for uptake by macrophages for 2 h. Then, the cells were collected and washed with PBS to remove free LDH NPs, followed by further culture in fresh medium. The cells were finally collected and washed with PBS at the time point of 0, 0.5, 1, 2, 4, or 8 h post incubation, and fixed in 4% PFA solution for FACS analysis.

Exchange of Internalized LDH NPs Between Macrophages

Intercellular exchange of LDH NPs between macrophage cells was examined by co-culturing two individually labeled macrophage populations. In brief, 25 μg/mL of LDH-FITC and LDH-CR NPs were separately added into RAW 264.7 macrophage cell suspensions for cellular uptake for 2 h, yielding two cell populations (each labeled with a specific dye), i.e., $M\Phi_{LDH-FITC}$ and $M\Phi_{LDH-CR}$. Two cell populations were then mixed at the equal cell number and then co-cultured for 4 h in fresh medium. The co-cultured cells were collected and fixed for FACS analysis, and cell images were taken using a Carl Zeiss LSM 510 confocal laser-scanning microscope (CLSM, Carl Zeiss MicroImaging GmbH, Germany). For comparison, two cell populations, i.e., $M\Phi_{LDH-FITC}$ and $M\Phi_{LDH-CR}$, were also cultured for 4 h separately in fresh medium and analyzed.

BMDC Maturation Induced by LDH-OVA NPs

At day 7 or 8, BMDCs were cultured in ultra-low attachment plates and pulsed with OVA (albumin from hen egg white, lyophilized powder, ≥98%, Grade VI; Sigma-Aldrich) or the equal amount of OVA in complex with LDH in RPMI 1640 medium (without GM-CSF) for 16 h. Cells were then harvested and washed, and stained with Alexa Fluor® 488 anti-mouse I-A/I-E Antibody (Clone 2G9; BioLegend) to determine dendritic cell maturation. Here LDH NPs with OVA were made by mixing them at the mass ratio of 2:1, at the concentration of 200 and 100 μg/mL, respectively.

SIINFEKL-Antigen Presentation in DC2.4 Cells Promoted by LDH NPs

After cell internalization, OVA antigen would be enzymatically degraded into the functional epitopes. OVA H-2Kb-restricted CTL epitope ($OVA_{257-264}$, SIINFEKL) would interact with MHC class I complexes, leading to the presentation of the MHC class I-functional epitope (like SIINFEKL) complex on the surface of DCs. To do this assay, DC 2.4 cells were cultured in 96 well cell culture plates, and pulsed with OVA in complex with LDH in RPMI 1640 medium (without GM-CSF) for 16 h. Then cells were harvested and washed, and stained with APC or PE anti-mouse H-2Kb of MHC class I bound to SIINFEKL antibody (Clone 25-D1.16; BioLegend) to determine the degree of epitope presentation (SIINFEKL/H-2Kb complexes) on DC2.4 cell surface.

B3Z CD8+ T Hybridoma Cell Activation

T cell priming can also indicate SIINFEKL epitope presented on the murine Kb MHC class I molecules (Karttunen et al., 1992). B3Z cell (kindly provided by A/Prof Mingnan Chen, University of Utah, United States), a CD8+ T-cell hybridoma, induces β-galactosidase (β-gal) production through T cell receptor interaction with SIINFEKL/H-2Kb complexes. To do this assay, DC 2.4 cells were cultured in 96 well plates at a density of 1×10^5 cells/mL, and were pulsed with OVA or LDH-OVA at a designed concentration for 16 h. After washing with PBS, the same number of B3Z cells were added to DC 2.4 cells. After 24 h co-culture, cells were washed and incubated with lysis buffer and chlorophenol red β-galactoside for 4 h. After stopping the reaction by EDTA and glycine, the OD value of the buffer was measured at 570 nm with that at 635 nm as the reference, in order to measure the activation degree of B3Z cells, which also reflects the cross-presentation amount of SIINFEKL/H-$2K^b$ complexes on DC 2.4 cells.

Statistical Analysis

Data presented as mean ± standard error of the mean (SEM) were analyzed by one-way analysis of variance (ANOVA), followed by multiple comparisons using Tukey's test within GraphPad Prism software. A p-value <0.05 was considered statistically significant. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and $^{****}p < 0.0001$.

RESULTS

Physicochemical Features of LDH-FITC and LDH-CR NPs

Both LDH-FITC and LDH-CR NPs were well dispersed in aqueous suspensions, showing a moderate particle

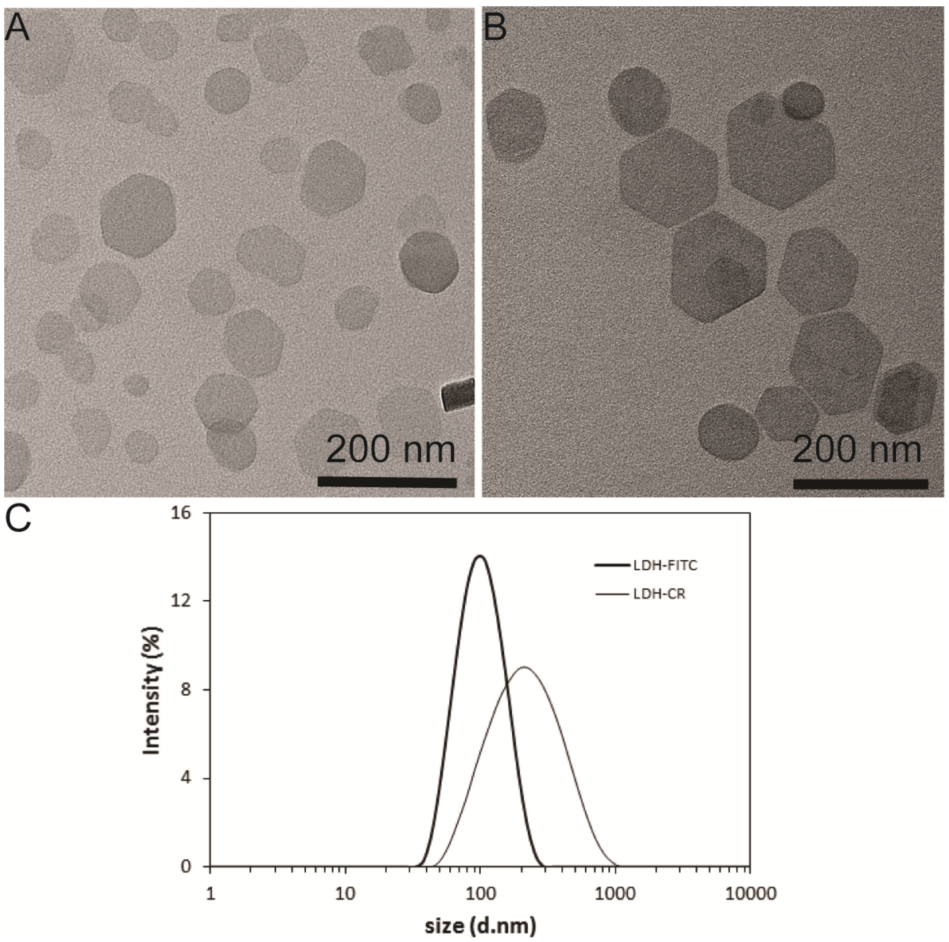

FIGURE 1 | Layered double hydroxide (LDH) NP Physicochemical Feautres. TEM image of LDH-FITC **(A)** and LDH-CR **(B)** NPs; and size distribution by intensity for LDH-FITC and LDH-CR NPs **(C)** in aqueous solution.

size distribution (**Figures 1A–C**). The equivalent mean hydrodynamic diameter for LDH-FITC and LDH-CR was 106 and 250 nm with the polydispersity index (PDI) of 0.132 and 0.255, respectively. Most LDH-FITC NPs were distributed within a range of 40–220 nm, while LDH-CR NPs were in 60–800 nm. The larger LDH-CR NPs may result from the longer heating time in the autoclave and the slight aggregation due to the higher CR loading. The estimated FITC was 10% of the anion exchange capacity and CR was ∼20%. The higher CR loading may also facilitate the LDH-CR crystallite growth at a relatively quicker rate than the lower FITC loading (**Figures 1A,B**; Xu and Braterman, 2003). In addition, FTIR spectra and XRD patterns confirm the layered structure of LDH-FITC and LDH-CR (**Supplementary Figure S1**), with Cl^- as the most abundant anion in the LDH interlayer.

Interestingly, when LDH-FITC and LDH-CR NP suspensions were mixed with culture medium separately, the average hydrodynamic particle size was increased by about 2 times (**Supplementary Figure S2**), suggesting slight aggregation caused by serum proteins through the bridging effect, as reported previously in our group (Gu et al., 2015). This slight aggregation does not severely affect the cellular uptake by immune cells, as presented shortly.

Immune Cell's Uptake Kinetics

The uptake kinetics of LDH-FITC NPs by immune cells (macrophages and DCs) was quantified by measuring the fluorescence intensity of each cell using the flow cytometry. As shown in **Figure 2** for macrophage uptake, the mean fluorescence intensity (MFI) was increased with the incubation time from 0.5 to 8 h at the LDH-FITC concentration of 5 and 25 μg/ml, respectively, indicating the cellular uptake is time-dependent. Interestingly, at both LDH-FITC doses, MFI increase was relatively quicker in the first 4 h than in the subsequent 4 h, as previously observed for the uptake of many other cells (Xu et al., 2008b; Oh et al., 2009; Wong et al., 2010).

Relatively, the uptake amount (MFI) at the low dose of LDH-FITC NPs (5 μg/ml) is much smaller than that at the higher dose (25 μg/ml) at all incubation time points, reflecting the cellular uptake is dose-dependent. In particular, FITC-positive cells reached 85–95% just after incubation for 1–2 h at the higher dose, i.e., almost all cells took up an enough amount of

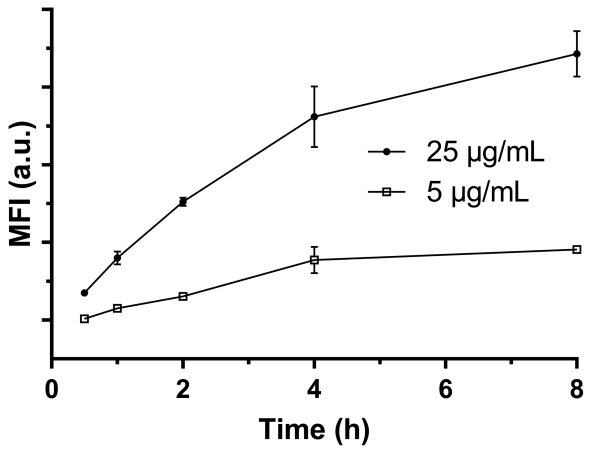

FIGURE 2 | The mean fluorescent intensity (MFI) of positive viable RAW 264.7 cells after uptake of LDH-FITC NPs at 37°C in a 5% CO_2 incubator.

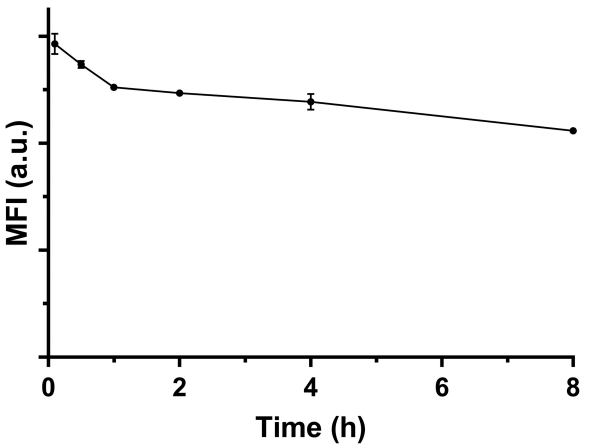

FIGURE 3 | The MFI of FITC-positive viable cells changing with the incubation time in fresh medium at 37°C.

LDH-FITC in 1–2 h (**Supplementary Figure S3**) to distinguish themselves from un-treated cells. This thus indicates that the uptake of LDH-FITC NPs by macrophage cells is very rapid, and in consistence with our previous findings for other cells (Xu et al., 2008b; Musumeci et al., 2010). Similarly, LDH-CR NPs were also quickly taken up by macrophage cells (**Supplementary Figure S4**; Oh et al., 2009). The quick cellular uptake of LDH NPs can be largely attributed to the quick endosomal escape of LDH NPs during endocytosis, as reported previously (Ladewig et al., 2010; Gu et al., 2011).

As further shown in **Supplementary Figure S5**, the freshly obtained BMDCs took up LDH-FITC NPs also quickly, in a dose- and time-dependent way, as reported previously for BMDCs (Li et al., 2010) and other mammalian cells (Xu et al., 2008b; Oh et al., 2009).

No Exocytosis of Internalized LDH NPs by Macrophage Cells

Our results indicate that there were ∼90% FITC-positive macrophage cells after culture for 2 h at the LDH-FITC dose of 25 μg/ml (**Supplementary Figure S3**), as also shown as the point at 0 h in **Supplementary Figure S6**. Thus, these cells took up an essential amount of LDH-FITC NPs. After replacement of LDH-FITC containing medium with fresh medium, these LDH-FITC-positive cells were further cultured to examine whether they release the internalized LDH-FITC NPs.

As shown in **Figure 3**, the relative MFI was reduced from 100 to 72% (28% reduction) after 8 h incubation in fresh culture medium. In particular, the relative MFI decreased from 100 to 86% (14% reduction) in the first 1 h, much more quickly than in the subsequent 7 h (14%). There are a few possible factors that contribute to the reduction of LDH-FITC NPs in each cell. The first factor is cell division. The total cell number may increase by ∼30% through division after 8 h incubation supposing that the cycle time of RAW 264.7 cells is 15–20 h. We believe that the cell division would largely explain the MFI reduction during this 8 h. The second factor is the release of the fluorescent tag (FITC) from the LDH interlayer, which may be degraded by the cell or diffuse out of the cell. If FITC release takes place in later endosome, the free FITC may also be quenched in the low pH environment. FITC release and quench may be responsible for the quick MFI reduction in the first 1 h just after cells were treated. The third factor is cellular exocytosis (release), which seems to contribute little to the MFI reduction.

Based on this test and analysis, we may conclude that macrophage cells do not obviously exocytosize the internalized LDH NPs, but keep them within the cells and passage to the next generation. As reported previously, iron oxide nanoparticles (IONPs) internalized by cells are retained within the cells and passaged to the cells in subsequent 3–4 generations (Gu et al., 2005).

LDH NP Intercellular Exchange Between Macrophage Cells

As shown in **Figures 4A,B**, and **Supplementary Table S1**, the fluorescence intensity of LDH-FITC and LDH-CR NP-treated cells ($M\Phi_{LDH-FITC}$ and $M\Phi_{LDH-CR}$) was increased from 7,300 ($M\Phi_{control}$) to 176,000 ($M\Phi_{LDH-FITC}$) and from 2,400 ($M\Phi_{control}$) to 50,000 ($M\Phi_{LDH-CR}$) after 2-h uptake and 4-h post-incubation (**Supplementary Table S1**), with ∼80% cells being fluorescence positive (**Table 1**). When $M\Phi_{LDH-FITC}$ and $M\Phi_{LDH-CR}$ cells were mixed in the equal number and the fluorescence intensity was quickly measured. The cytometry profile was their simple combination (**Figures 4A–C**), i.e., half of their individual positive cell percentage, i.e., 39% for each population [**Table 1**, $M\Phi_{LDH-FITC} + M\Phi_{LDH-CR}$ (0 h) and **Figure 4C**], without obvious change for the intensity of these two cell populations (**Supplementary Table S1**).

After 4 h incubation of the mixed cells, two populations moved into the cross to close each other (**Figure 4D** and **Supplementary Figure S7**). For example, the FITC intensity of two cell populations [$M\Phi_{LDH-FITC} + M\Phi_{LDH-CR}$ (4 h)] was 26,800/64,200, in sharp contrast to 12,400/173,000

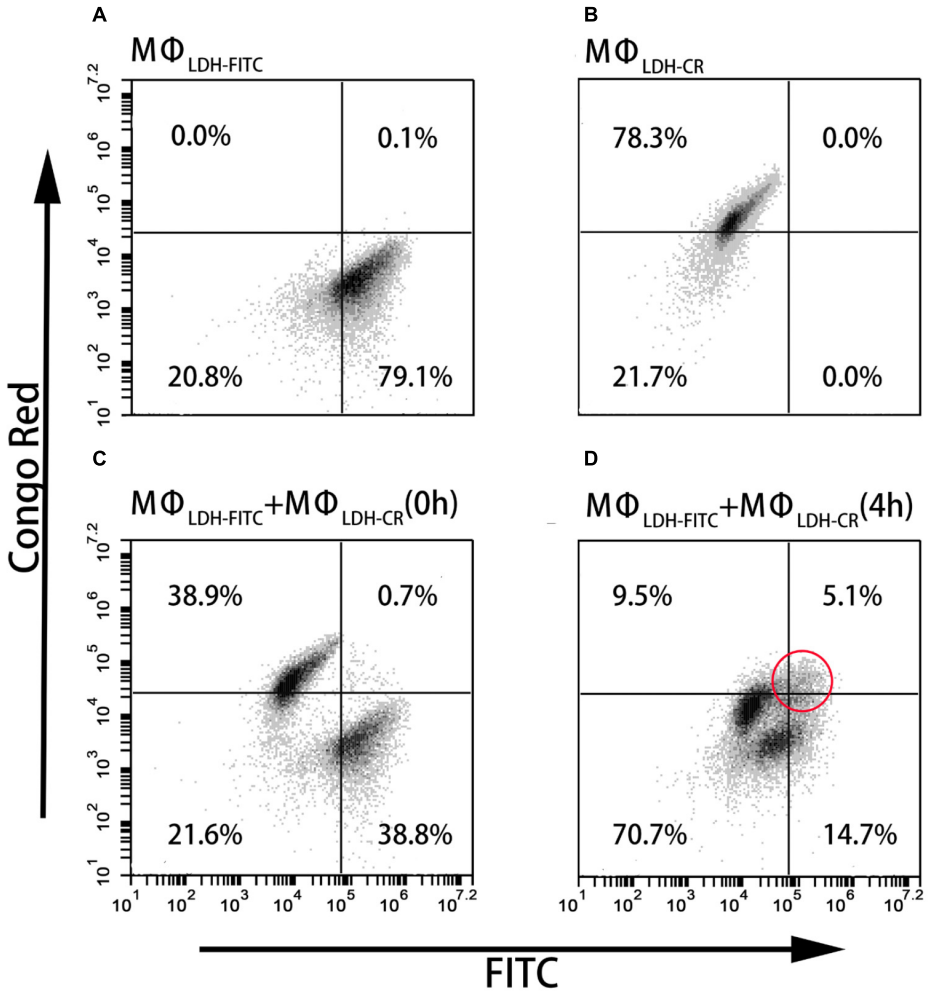

FIGURE 4 | FACS dot plots showing the cell staining. **(A–D)** The dot plots showing Flow cytometric analysis for intercellular exchange of LDH NP by macrophages. **(A)** LDH-FITC macrophages ($M\Phi_{LDH-FITC}$); **(B)** LDH-CR macrophages ($M\Phi_{LDH-CR}$); **(C)** Quick mixture of LDH-FITC macrophages and LDH-CR macrophages [$M\Phi_{LDH-FITC}$ + $M\Phi_{LDH-CR}$ (0 h)]; and **(D)** 4-h mixture of LDH-FITC macrophages and LDH-CR macrophages [$M\Phi_{LDH-FITC}$ + $M\Phi_{LDH-CR}$ (4 h)].

TABLE 1 | Positive macrophage cell (MΦ) percentage labeled with FITC and CR.

Percentage (%)	$M\Phi_{control}$	$M\Phi_{LDH-FITC}$	$M\Phi_{LDH-CR}$	$M\Phi_{LDH-FITC}$ + $M\Phi_{LDH-CR}$ (0 h)	$M\Phi_{LDH-FITC}$ + $M\Phi_{LDH-CR}$ (4 h)
$FITC^+$ cell	0	79.1	0	38.8	14.7
CR^+ cell	0	0	78.3	38.9	9.5
$FITC^+ + CR^+$ cell	0	0.1	0	0.7	5.1

[$M\Phi_{LDH-FITC}$ + $M\Phi_{LDH-CR}$ (0 h)] (**Supplementary Table S1**). Similarly, the CR intensity was 4,500/18,700 vs. 3,400/49,700, respectively (**Supplementary Table S1**). Very remarkably, there were 5.1% cells being both FITC-positive and CR-positive (**Figure 4D**, indicated with the red circle), while the percentage of only FITC-positive and only CR-positive cells was significantly reduced to 14.7 and 9.5%, respectively [**Table 1**, $M\Phi_{LDH-FITC}$ + $M\Phi_{LDH-CR}$ (4 h)]. The histograms indicate that $M\Phi_{LDH-FITC}$ + $M\Phi_{LDH-CR}$ (4 h) cells obviously shift in both FITC and Congo red channels (**Supplementary Figure S7**), and confirmed that some cells in $M\Phi_{LDH-FITC}$ + $M\Phi_{LDH-CR}$ (4 h) population contained both LDH-FITC and LDH-CR NPs, clearly showing that macrophage cells exchange the LDH NPs with each other. The nanoparticle exchange may occur via the possible mechanisms for the transfer of antigens between APCs, such as synapse (Mittelbrunn and Sanchez-Madrid, 2012), via tunneling nanotubes (TNT) (Domhan et al., 2011), or through gap junctions (Yewdell and Dolan, 2011).

This exchange has been also captured in the fluorescence image. As shown in **Figure 5D**, the arrow indicates that the macrophage cell has both LDH-FITC and LDH-CR NPs inside. These data thus reveal that the macrophage cells exchange the

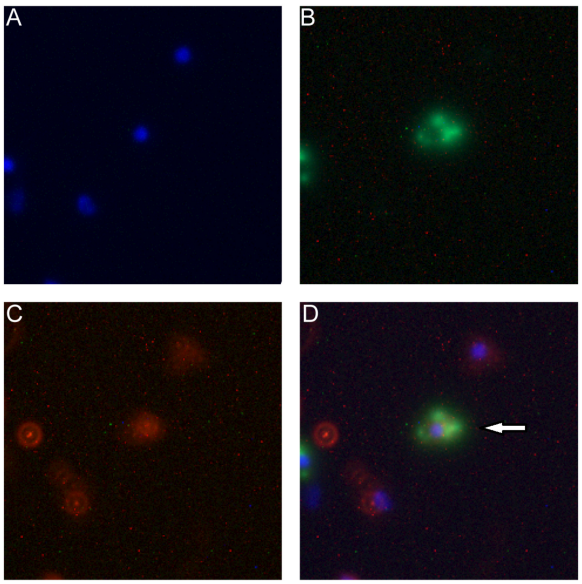

FIGURE 5 | Fluorescence microscope images of macrophage cells being both FITC-positive (green) and LDH-CR (4 h). $M\Phi_{LDH\text{-}FITC} + M\Phi_{LDH\text{-}CR}$-positive (red) after 4 h incubation [M Channels: **(A)** Nuclei (blue); **(B)** LDH-FITC; **(C)** LDH-CR; **(D)** Merged]. The arrow pointing the cell with both LDH-FITC and LDH-CR NPs inside.

internalized NPs with their neighbors, which appeared to occur even in suspension when the two populations of cells were just mixed and contacted within a minute, as there were 0.7% cells being already both FITC-positive and CR-positive [**Table 1**, $M\Phi_{LDH-FITC} + M\Phi_{LDH-CR}$ (0 h)].

BMDC Maturation Promoted by LDH-OVA

High expression of MHC class II complexes on the DC surface, i.e., DC maturation, is very critical for generation of a high level of antigen-specific antibody (Kukutsch et al., 2000). To demonstrate the maturation effect of LDH-OVA vaccine formulation, BMDCs cultured at day 7 or 8 were collected as the target DCs. LDH-OVA was formulated at the LDH:OVA mass ratio of 2:1, and BMDCs were then exposed to culture medium containing this LDH-OVA formulation for 16 h. The I-A/I-E antibody was used to distinguish two kinds of DC subpopulations, i.e., MHC II high and MHC II low (**Supplementary Figure S8**), which are representative for mature and immature DCs according to the previous study (Kukutsch et al., 2000).

Our data show that there was a significant increase in terms of mature DCs when DCs were stimulated by the LDH-OVA formulation. As shown in **Figure 6A**, the MFI of MHC II high DC population treated with LDH-OVA was significantly higher than that of the blank control and OVA only-activated DC group. Consistently, the mature DC was up to 54.2% when LDH-OVA was used to stimulate DCs, significantly higher than the control group (35.4%) and OVA-stimulated group (48.0%) (**Figure 6B**). Thus LDH NPs significantly promote the maturation of DCs, as reported previously (Li et al., 2010).

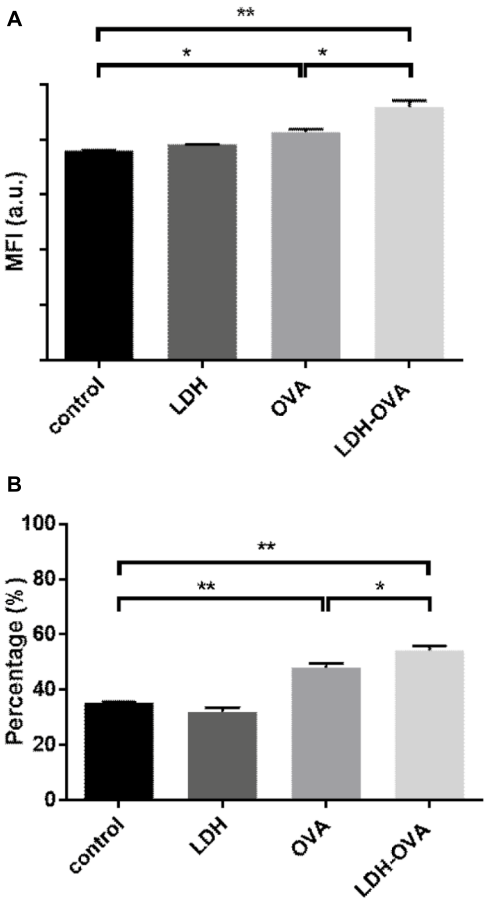

FIGURE 6 | Layered double hydroxide-ovalbumin (LDH-OVA) induces BMDC maturation. The MFI **(A)** and percentage **(B)** of MHC class II high population. The data shown are representative for three independent experiments. Data are the mean ± SEM. $^*p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA, with a Tukey's multiple comparison test).

Enhanced Antigen Cross-Presentation Promoted by LDH NPs

As previously presented, blank LDH NPs are readily taken up by macrophage cells and DCs, which can be used to carry the target antigens and facilitate their cellular uptake, such as BSA (Chen et al., 2016) and OVA. After internalization, OVA antigen is probably dissociated from LDH-OVA particles either in later endosome or cytoplasm, and then enzymatically degraded into the functional epitope. This epitope interacts with MHC class I complexes, leading to the presentation of the MHC class I-functional epitope (like SIINFEKL) complexes on the surface of DCs.

In this research, 25-D1.16 antibody was used to specifically bind with the complex (SIINFEKL/H-$2K^b$) to confirm and quantify the antigen cross-presentation through the MHC class I pathway, which is necessary for inducing the formation of anti-tumor CTL CD8+ T cells (Burgdorf et al., 2007). As shown in **Figure 7A**, LDH-OVA vaccine significantly enhanced the presentation of SIINFEKL/MHC I complexes on the surface

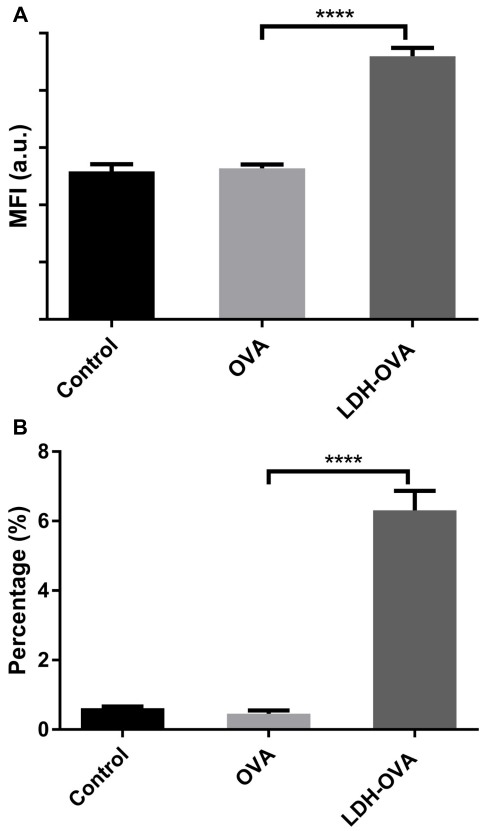

FIGURE 7 | Enhanced presentation of SIINFEKL/MHC I complexes in DC 2.4 cells by LDH-facilitated OVA delivery. The cell MFI **(A)** and the percentage **(B)** of cells expressing SIINFEKL/H-2Kb complexes. Data are the mean ± SEM. ****$p < 0.0001$ (one-way ANOVA, with a Tukey's multiple comparison test).

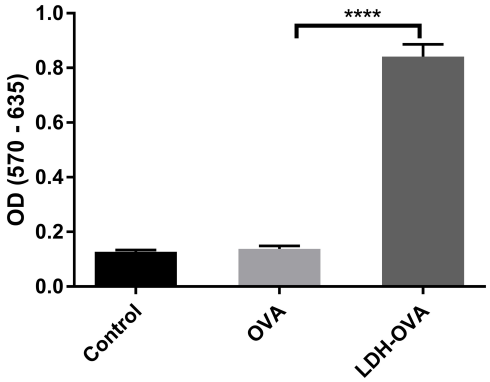

FIGURE 8 | Activation of B3Z cells after co-incubation with LDH-OVA pulsed DC 2.4. Data are the mean ± SEM. ****$p < 0.0001$ (one-way ANOVA, with a Tukey's multiple comparison test).

of DC 2.4 in terms of the MFI, with up to 6.3% of DC 2.4 presenting complexes, in sharp contrast with nearly no antigen presented in DC 2.4 cells treated with OVA only (0.45%) and control medium (0.62%) (**Figure 7B**). The higher antigen-complex presentation on the DC surface may be largely attributed to the promoted cellular uptake and moreover, the enhanced subsequent processes, such as enzymatic degradation of OVA to epitope with the help of LDH NPs, as well as formation of MHC I-epitope complexes through the cytosolic pathway with endoplasmic reticulum (ER) or phagosomal loading (Joffre et al., 2012). Moreover, as shown in **Supplementary Figure S9**, LDH-SIINFEKL vaccine resulted in high SIINFEKL presentation by DCs which is comparable with the presentation using free SIINFEKL as the positive control. Free SIINFEKL peptide is well known to be readily loaded onto MHC class I after exogenous loading/incubation with DC's or APCs (Cho et al., 2016).

Enhanced T Cell Priming by LDH-OVA-Treated DCs

T cell priming can also indicate whether the antigen is cross-presented on the DC surface in the form of MHC class I-epitope complexes. Thus, B3Z cell, a CD8+ T-cell hybridoma, was chosen to specifically recognize SIINFEKL epitope presented on the murine Kb MHC class I molecules (Karttunen et al., 1992) and determine the T cell priming extent. The priming of the SIINFEKL epitope to B3Z cells induces β-galactosidase (β-gal) synthesis by B3Z cells. The induced β-gal amount thus quantifies the cross-presentation of SIINFEKL/H-2K^b complexes on DC cells and the degree of T cell activation. In this research, an equal number of B3Z and DCs (LDH-OVA stimulated) were co-cultured for 24 h, and the OD value, i.e., the β-gal amount produced by B3Z, was measured in a plate reader. As shown in **Figure 8**, the OD value of lyzed B3Z cells that were co-cultured with LDH-OVA pulsed DCs was significantly higher than that of other two control groups, indicating that B3Z cells were significantly activated and further confirming that the OVA epitope was successfully cross-presented on DC 2.4 cells via LDH NP adjuvants.

DISCUSSION

Layered double hydroxide nanomaterials are reported to significantly promote the immune responses in mice model and show a high promise as effective nano-adjuvants (Li et al., 2011; Wang et al., 2014; Williams et al., 2014). In particular, our groups have demonstrated that LDH NPs are able to induce both high-level antibody and cellular immune responses for antibacterial and anti-tumor treatment (Yan et al., 2014; Chen et al., 2016). Apparently, the adjuvanticity of LDH NPs is related to the particle size and composition, the mass ratio of LDH: antigen, and the dose injected, which have been reported (Williams et al., 2014; Yan et al., 2014; Chen et al., 2016). In principle, the activity is largely determined by the effects of LDH NPs on the biological processes of immune cells, including long-term stimulation (depot effect), cellular uptake, APC maturation, antigen processes within APCs and the antigen presentation on the APC surface, as well as the activation of target T and B cells, which have been well investigated in the current research, together with our previous work (Yan et al., 2014; Chen et al., 2016).

When LDH-antigen NPs are injected subcutaneously, these NPs form a loosely aggregated lump, which then causes a so-called depot effect, i.e., long-term stimulation. For example, the lump of LDH NP-adjuvanted vaccine was found to last for ∼1 month beneath the skin, giving a higher and sustained level of specific antibody (Chen et al., 2016, 2018). Thus, the depot effect is beneficial to the long-term immune responses (Mckee et al., 2007; Henriksen-Lacey et al., 2010). Moreover, the lump recruits many inflammatory cells (Aimanianda et al., 2009; Chen et al., 2018), thus LDH-antigen NPs on the lump surface can be readily taken up by or facilitate the delivery of antigen (such as OVA) to these immune cells. The current research has further confirmed that LDH NPs are readily taken up by macrophage cells (**Figure 2**) and BMDCs (**Supplementary Figure S5**), which is also supported by previous reports (Li et al., 2010; Wang et al., 2014). As revealed elsewhere, this facilitation to cellular uptake results from the quick endosome escape (Choy et al., 2004; Xu et al., 2008b). As weakly alkaline LDH NPs are partly dissolved in the slightly acidic endosome, so the ion concentration increases and the enhanced osmotic pressure inside the endosome leads to water influx and bursts the endosome, releasing the LDH NPs into the cytoplasm (Xu et al., 2008b; Gu et al., 2011). Therefore, antigen is mostly associated with LDH NPs after endosome escape and then possibly processed to load with MHC I molecules through the cross-presentation pathway. This process is very much different from the case using polymeric nanoparticles to adjuvant antigens through lysosomal pathway (Lai et al., 2007, 2008; Fernando et al., 2010).

The most potent APCs are immunologically competent dendritic cells (DCs), while their ability to regulate immunity is dependent on their maturation (Banchereau and Steinman, 1998). After the LDH-antigen vaccine is injected subcutaneously, immature DCs are recruited to the site of inflammation in peripheral tissues, and take up LDH-antigen NPs (Waeckerle-Men et al., 2004; Chen et al., 2016, 2018). In this research, we found that LDH NPs assist OVA to mature DCs by promoting significantly more MHC II complexes on the DCs' surface (**Figure 6**), in coordination with the activation signals received from the surrounding cytokines and chemokines, costimulatory molecules and proteases (Li et al., 2010; Williams et al., 2014). On the other hand, the exogenous OVA antigens are quickly delivered to cytoplasm by LDH NPs, and processed into epitopes for complexing with MHC I molecules (cross-presentation), which is also benefited from LDH's quick endosome escape

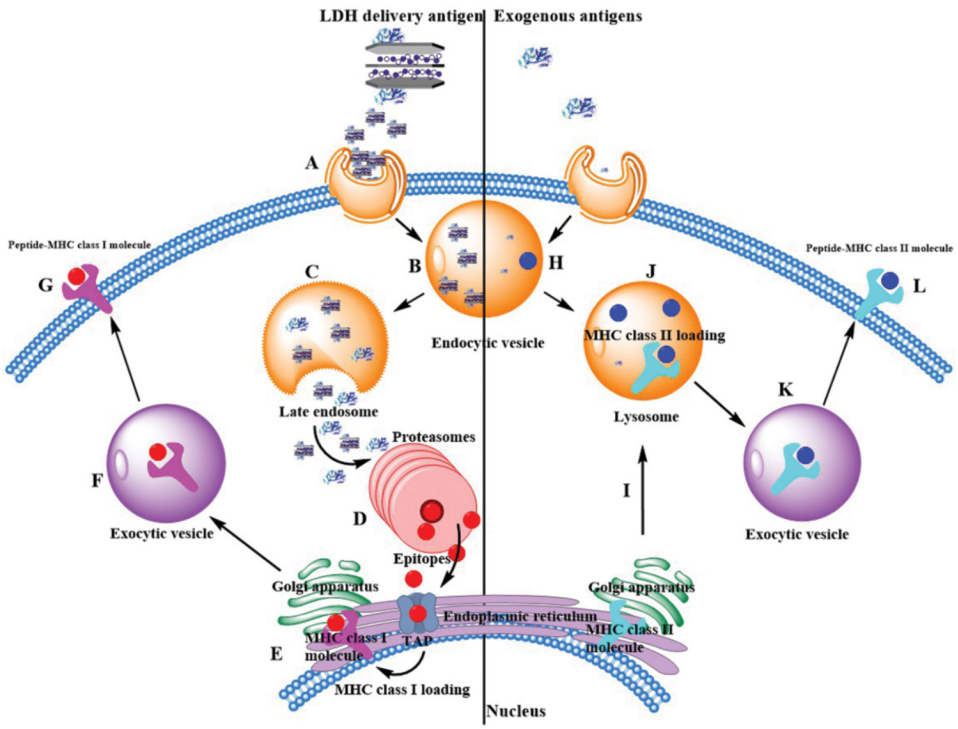

FIGURE 9 | Exogenous antigen delivery mechanism through MHC II pathway by itself or through MHC I cross-presentation pathway by LDH NPs. **(Left)** LDH-based vaccine through endocytosis (Step A) is internalized into the endosome (Step B). Through the endosome escape, free antigen, and LDH-based vaccine are released into the cytoplasm (Step C), and antigens are enzymatically degraded into functional epitopes within proteasomes (Step D). With the help of transporter associated with antigen processing (TAP), epitopes are transferred into endoplasmic reticulum (ER), where they are loaded with MHC class I molecules (Step E). Afterward, these complexes are transferred to exocytic vesicle by Golgi apparatus (Step F). Finally, MHC class I complexes present these epitopes on the surface of antigen presentation cells (Step G). **(Right)** Generally exogenous antigens are intracellular internalization in endocytic vesicles, such as acidic intracellular compartments. Antigens are degraded into epitopes (Step H). Then endocytic vesicles fuse with lysosome, where epitopes are loaded with MHC class II molecules (Step J). Note that MHC class II molecules are made inside the ER and transferred to lysosome by Golgi apparatus (Step I). Finally, these epitopes are presented with MHC class II complexes on the cell surface (Step L) by exocytic vesicles (Step K).

(as schematically shown in **Figure 9**). Meanwhile, LDH-antigen NPs could also attract the proteasome and enzymatic proteases. These proteases on the LDH surface may more efficiently process the adjacent antigens into antigenic epitope. Moreover, short-chain epitope is more easily released from the LDH surface, which may quickly form more epitope-loaded MHC class II and I complexes and their subsequent (cross)-presentation. This postulated mechanism may be supported by the enhanced MHC class II high population and more SIINFEKL/MHC I complexes in LDH-OVA group (**Figures 6**, **7**), respectively. Therefore, the mature DCs have significantly enhanced presentation of antigen-loaded MHC class I complexes on the cell surface upon the stimulation of the LDH-antigen vaccine.

A more interesting issue is that immune cells (such as APCs) that take up LDH-antigen NPs may exchange these NPs with surrounding immature cells (including macrophages and DCs) at the site of injection, during the circulation in blood/lymph systems, and in the lymphoid nodes. As demonstrated in this research, macrophage cells exchanged their internalized LDH NPs with each other during *in vitro* culture (**Figures 4**, **5**). In such a way, DCs that take up LDH-antigen NPs at the injection/inflammatory site may transfer these LDH NPs to the surrounding immature DCs, thus "infect" and activate these immature DCs to mature (epitope/MHC class II presentation) and present epitope/MHC class I complexes (cross-presentation). As reported recently, cellular communication by exchanging materials may occur via synapse (Mittelbrunn and Sanchez-Madrid, 2012), tunneling nanotubes (TNT) (Domhan et al., 2011), or gap junctions (Yewdell and Dolan, 2011). Subsequently, this exchange induces a high level of specific antibody by stimulating B cells and activates more potent cytotoxic T cells for cell-mediated immune response, as reported in our previous paper (Yan et al., 2014) and this research (**Figure 8**), leading to the remarkable improvement in the immune responses.

CONCLUSION

In summary, we report that APCs (such as macrophages and DCs) can take up LDH NPs efficiently, and more significantly macrophages exchange the internalized LDH NPs with surrounding ones. We also report that the internalized LDH-antigen NPs can significantly facilitate the maturation of immature DCs and enhance the antigen cross-presentation of MHC I complexes on the DC surface. The high adjuvanticity of LDH NPs may be attributed to specific properties of LDH materials, such as the weak alkalinity for endosome escape and capability of co-adsorbing enzymes on the surface for enzymatic degradation. These findings may provide some guidelines for design new adjuvants for next generation vaccines.

AUTHOR CONTRIBUTIONS

ZX and LL designed the current experiments in consultation with WG and BR. SY conducted most of the experiments and collected and analyzed the data. KX assisted in the experiments and data collection. SY and ZX wrote the manuscript. LL, KX, WG, and BR contributed to the revisions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01060/full#supplementary-material

REFERENCES

Aimanianda, V., Haensler, J., Lacroix-Desmazes, S., Kaveri, S. V., and Bayry, J. (2009). Novel cellular and molecular mechanisms of induction of immune responses by aluminum adjuvants. *Trends Pharmacol. Sci.* 30, 287–295. doi: 10.1016/j.tips.2009.03.005

Banchereau, J., and Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature* 392, 245–252. doi: 10.1038/32588

Braterman, P., Xu, Z., and Yarberry, F. (2004). *In Handbook of Layered Materials*, ed. P. K. Dutta, New York, NY: Marcel Dekker.

Burgdorf, S., Kautz, A., Bohnert, V., Knolle, P. A., and Kurts, C. (2007). Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science* 316, 612–616. doi: 10.1126/science.1137971

Chen, W., Zhang, B., Mahony, T., Gu, W., Rolfe, B., and Xu, Z. P. (2016). Efficient and durable vaccine against intimin beta of diarrheagenic *E. Coli* induced by clay nanoparticles. *Small* 12, 1627–1639. doi: 10.1002/smll.201503359

Chen, W., Zuo, H., Li, B., Duan, C., Zhang, B., Rolfe, B., et al. (2018). Clay nanoparticles elicit long-term immune responses by forming biodegradable depots for sustained antigen stimulation. *Small* 14:1704465. doi: 10.1002/smll.201704465

Cheng, K., El-Boubbou, K., and Landry, C. C. (2012). Binding of HIV-1 gp120 glycoprotein to silica nanoparticles modified with CD4 glycoprotein and CD4 peptide fragments. *ACS Appl. Mater. Interfaces* 4, 235–243. doi: 10.1021/am2013008

Cho, S., Dong, S., Parent, K. N., and Chen, M. (2016). Immune-tolerant elastin-like polypeptides (iTEPs) and their application as CTL vaccine carriers. *J. Drug Target* 24, 328–339. doi: 10.3109/1061186x.2015.1077847

Choy, J. H., Jung, J. S., Oh, J. M., Park, M., Jeong, J., Kang, Y. K., et al. (2004). Layered double hydroxide as an efficient drug reservoir for folate derivatives. *Biomaterials* 25, 3059–3064. doi: 10.1016/j.biomaterials.2003.09.083

Domhan, S., Ma, L. L., Tai, A., Anaya, Z., Beheshti, A., Zeier, M., et al. (2011). Intercellular communication by exchange of cytoplasmic material via

Tunneling nano-tube like structures in primary human renal epithelial cells. *PLoS One* 6:e21283. doi: 10.1371/journal.pone.0021283

Fan, Y., and Moon, J. J. (2015). Nanoparticle drug delivery systems designed to improve cancer vaccines and immunotherapy. *Vaccines* 3, 662–685. doi: 10.3390/vaccines3030662

Fernando, L. P., Kandel, P. K., Yu, J. B., McNeill, J., Ackroyd, P. C., and Christensen, K. A. (2010). Mechanism of cellular uptake of highly fluorescent conjugated polymer nanoparticles. *Biomacromolecules* 11, 2675–2682. doi: 10.1021/bm1007103

Gu, H. W., Xu, K. M., Yang, Z. M., Chang, C. K., and Xu, B. (2005). Synthesis and cellular uptake of porphyrin decorated iron oxide nanoparticles—a potential candidate for bimodal anticancer therapy. *Chem. Commun.* 4270–4272. doi: 10.1039/b507779f

Gu, Z., Rolfe, B. E., Thomas, A. C., Campbell, J. H., Lu, G. Q., and Xu, Z. P. (2011). Cellular trafficking of low molecular weight heparin incorporated in layered double hydroxide nanoparticles in rat vascular smooth muscle cells. *Biomaterials* 32, 7234–7240. doi: 10.1016/j.biomaterials.2011.05.083

Gu, Z., Zuo, H. L., Li, L., Wu, A. H., and Xu, Z. P. (2015). Pre-coating layered double hydroxide nanoparticles with albumin to improve colloidal stability and cellular uptake. *J. Mater. Chem. B* 3, 3331–3339. doi: 10.1039/c5tb00248f

Guo, H. C., Feng, X. M., Sun, S. Q., Wei, Y. Q., Sun, D. H., Liu, X. T., et al. (2012). Immunization of mice by hollow mesoporous silica nanoparticles as carriers of porcine circovirus type 2 ORF2 Protein. *Virol. J.* 9:108. doi: 10.1186/1743-422X-9-108

Hamdy, S., Molavi, O., Ma, Z. S., Haddadi, A., Alshamsan, A., Gobti, Z., et al. (2008). Co-delivery of cancer-associated antigen and toll-like receptor 4 ligand in PLGA nanoparticles induces potent CD8(+) T cell-mediated anti-tumor immunity. *Vaccine* 26, 5046–5057. doi: 10.1016/j.vaccine.2008.07.035

He, Q., Mitchell, A., Morcol, T., and Bell, S. J. D. (2002). Calcium phosphate nanoparticles induce mucosal immunity and protection against herpes simplex virus type 2. *Clin. Diagn. Lab. Immunol.* 9, 1021–1024. doi: 10.1128/Cdli.9.5.1021-1024.2002

Henriksen-Lacey, M., Bramwell, V. W., Christensen, D., Agger, E. M., Andersen, P., and Perrie, Y. (2010). Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. *J. Control. Release* 142, 180–186. doi: 10.1016/j.jconrel.2009.10.022

Joffre, O. P., Segura, E., Savina, A., and Amigorena, S. (2012). Cross-presentation by dendritic cells. *Nat. Rev. Immunol.* 12, 557–569. doi: 10.1038/nri3254

Karttunen, J., Sanderson, S., and Shastri, N. (1992). Detection of rare antigen-presenting cells by the Lacz T-Cell activation assay suggests an expression cloning strategy for T-Cell antigens. *Proc. Natl. Acad. Sci. U.S.A.* 89, 6020–6024.

Kukutsch, N. A., Rossner, S., Austyn, J. M., Schuler, G., and Lutz, M. B. (2000). Formation and kinetics of MHC class I-ovalbumin peptide complexes on immature and mature murine dendritic cells. *J. Invest. Dermatol.* 115, 449–453. doi: 10.1046/j.1523-1747.2000.00084.x

Ladewig, K., Niebert, M., Xu, Z. P., Gray, P. P., and Lu, G. Q. M. (2010). Efficient siRNA delivery to mammalian cells using layered double hydroxide nanoparticles. *Biomaterials* 31, 1821–1829. doi: 10.1016/j.biomaterials.2009.10.058

Lai, S. K., Hida, K., Chen, C., and Hanes, J. (2008). Characterization of the intracellular dynamics of a non-degradative pathway accessed by polymer nanoparticles. *J. Control. Release* 125, 107–111. doi: 10.1016/j.jconrel.2007.10.015

Lai, S. K., Hida, K., Man, S. T., Chen, C., Machamer, C., Schroer, T. A., et al. (2007). Privileged delivery of polymer nanoparticles to the perinuclear region of live cells via a non-clathrin, non-degradative pathway. *Biomaterials* 28, 2876–2884. doi: 10.1016/j.biomaterials.2007.02.021

Li, A., Qin, L. L., Wang, W. R., Zhu, R. R., Yu, Y. C., Liu, H., et al. (2011). The use of layered double hydroxides as DNA vaccine delivery vector for enhancement of anti-melanoma immune response. *Biomaterials* 32, 469–477. doi: 10.1016/j.biomaterials.2010.08.107

Li, A., Qin, L. L., Zhu, D., Zhu, R. R., Sun, J., and Wang, S. L. (2010). Signalling pathways involved in the activation of dendritic cells by layered double hydroxide nanoparticles. *Biomaterials* 31, 748–756. doi: 10.1016/j.biomaterials.2009.09.095

Lutz, M. B., Kukutsch, N., Ogilvie, A. L., Rossner, S., Koch, F., Romani, N., et al. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223, 77–92.

Mckee, A. S., Munks, M. W., and Marrack, P. (2007). How do adjuvants work? Important considerations for new generation adjuvants. *Immunity* 27, 687–690. doi: 10.1016/j.immuni.2007.11.003

Mittelbrunn, M., and Sanchez-Madrid, F. (2012). Intercellular communication: diverse structures for exchange of genetic information. *Nat. Rev. Mol. Cell Biol.* 13, 328–335. doi: 10.1038/nrm3335

Musumeci, A. W., Mortimer, G. M., Butler, M. K., Xu, Z. P., Minchin, R. F., and Martin, D. J. (2010). Fluorescent layered double hydroxide nanoparticles for biological studies. *Appl. Clay Sci.* 48, 271–279. doi: 10.1016/j.clay.2009.11.008

Oh, J. M., Choi, S. J., Lee, G. E., Kim, J. E., and Choy, J. H. (2009). Inorganic metal hydroxide nanoparticles for targeted cellular uptake through clathrin-mediated endocytosis. *Chem. Asian J.* 4, 67–73. doi: 10.1002/asia.200800290

Scheerlinck, J. P. Y., Gloster, S., Gamvrellis, A., Mottram, P. L., and Plebanski, M. (2006). Systemic immune responses in sheep, induced by a novel nano-bead adjuvant. *Vaccine* 24, 1124–1131. doi: 10.1016/j.vaccine.2005.09.009

Slingluff, C. L. Jr. (2011). The present and future of peptide vaccines for cancer: single or multiple, long or short, alone or in combination? *Cancer J.* 17, 343–350. doi: 10.1097/PPO.0b013e318233e5b2

Toes, R. E. M., Offringa, R., Blom, R. J. J., Melief, C. J. M., and Kast, W. M. (1996). Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. *Proc. Natl. Acad. Sci. U.S.A.* 93, 7855–7860. doi: 10.1073/pnas.93.15.7855

Waeckerle-Men, Y., Scandella, E., Uetz-Von Allmen, E., Ludewig, B., Gillessen, S., Merkle, H. P., et al. (2004). Phenotype and functional analysis of human monocyte-derived dendritic cells loaded with biodegradable poly(lactide-co-glycolide) microspheres for immunotherapy. *J. Immunol. Methods* 287, 109–124. doi: 10.1016/j.jim.2004.01.010

Wang, J., Zhu, R. R., Gao, B., Wu, B., Li, K., Sun, X. Y., et al. (2014). The enhanced immune response of hepatitis B virus DNA vaccine using SiO2@LDH nanoparticles as an adjuvant. *Biomaterials* 35, 466–478. doi: 10.1016/j.biomaterials.2013.09.060

Williams, G. R., Fierens, K., Preston, S. G., Lunn, D., Rysnik, O., De Prijck, S., et al. (2014). Immunity induced by a broad class of inorganic crystalline materials is directly controlled by their chemistry. *J. Exp. Med.* 211, 1019–1025. doi: 10.1084/jem.20131768

Wong, Y. Y., Markham, K., Xu, Z. P., Chen, M., Lu, G. Q., Bartlett, P. F., et al. (2010). Efficient delivery of siRNA to cortical neurons using layered double hydroxide nanoparticles. *Biomaterials* 31, 8770–8779. doi: 10.1016/j.biomaterials.2010.07.077

Xu, Z. P., and Braterman, P. S. (2003). High affinity of dodecylbenzene sulfonate for layered double hydroxide and resulting morphological changes. *J. Mater. Chem.* 13, 268–273. doi: 10.1039/b207540g

Xu, Z. P., Jin, Y., Liu, S., Hao, Z. P., and Lu, G. Q. (2008a). Surface charging of layered double hydroxides during dynamic interactions of anions at the interfaces. *J. Colloid Interface Sci.* 326, 522–529. doi: 10.1016/j.jcis.2008.06.062

Xu, Z. P., Niebert, M., Porazik, K., Walker, T. L., Cooper, H. M., Middelberg, A. P. J., et al. (2008b). Subcellular compartment targeting of layered double hydroxide nanoparticles. *J. Control. Release* 130, 86–94. doi: 10.1016/j.jconrel.2008.05.021

Xu, Z. P., Stevenson, G., Lu, C. Q., and Lu, G. Q. (2006a). Dispersion and size control of layered double hydroxide nanoparticles in aqueous solutions. *J. Phys. Chem. B* 110, 16923–16929. doi: 10.1021/jp062281o

Xu, Z. P., Stevenson, G. S., Lu, C. Q., Lu, G. Q., Bartlett, P. F., and Gray, P. P. (2006b). Stable suspension of layered double hydroxide nanoparticles in aqueous solution. *J. Am. Chem. Soc.* 128, 36–37. doi: 10.1021/ja056652a

Xu, Z. P., Zeng, Q. H., Lu, G. Q., and Yu, A. B. (2006c). Inorganic nanoparticles as carriers for efficient cellular delivery. *Chem. Eng. Sci.* 61, 1027–1040. doi: 10.1016/j.ces.2005.06.019

Yan, S. Y., Gu, W. Y., Zhang, B., Rolfe, B. E., and Xu, Z. P. (2018). High adjuvant activity of layered double hydroxide nanoparticles and nanosheets in

anti-tumour vaccine formulations. *Dalton Trans.* 47, 2956–2964. doi: 10.1039/c7dt03725b

Yan, S. Y., Rolfe, B. E., Zhang, B., Mohammed, Y. H., Gu, W. Y., and Xu, Z. P. (2014). Polarized immune responses modulated by layered double hydroxides nanoparticle conjugated with CpG. *Biomaterials* 35, 9508–9516. doi: 10.1016/j.biomaterials.2014.07.055

Yewdell, J. W., and Dolan, B. P. (2011). IMMUNOLOGY Cross-dressers turn on T cells. *Nature* 471, 581–582. doi: 10.1038/471581a

Zhang, Q., Zhu, Y. H., Xu, H. B., and Yang, X. L. (2015). Multifunctional nanoparticle-based adjuvants used in cancer vaccines. *Prog. Chem.* 27, 275–285. doi: 10.7536/Pc141007

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