CANCER NANOTHERANOSTICS: WHAT HAVE WE LEARNED SO FAR?

EDITED BY: João Conde, Pedro Viana Baptista, Jesús M. De La Fuente and Furong Tian

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CANCER NANOTHERANOSTICS: WHAT HAVE WE LEARNED SO FAR?

Topic Editors:
João Conde, Massachusetts Institute of Technology and Harvard-MIT Division for Health Sciences and Technology, USA
Pedro Viana Baptista, Universidade Nova de Lisboa, Portugal
Jesús M. De La Fuente, Universidad de Zaragoza, Spain
Furong Tian, Dublin Institute Technology, Ireland

The beauty of Nanotheranostics world.
Image by João Conde created with Wordle (http://www.wordle.net).

After a quarter of century of rapid technological advances, research has revealed the complexity of cancer, a disease intimately related to the dynamic transformation of the genome. However, the full understanding of the molecular onset of this disease is still far from achieved and the search for mechanisms of treatment will follow closely.

It is here that Nanotechnology enters the fray offering a wealth of tools to diagnose and treat cancer.

In fact, the National Cancer Institute predicts that over the next years, nanotechnology will result in important advances in early detection, molecular imaging, targeted and multifunctional therapeutics, prevention and control of cancer. Nanotechnology offers numerous tools to diagnose and treat cancer, such as new imaging agents, multifunctional devices capable of overcome

biological barriers to deliver therapeutic agents directly to cells and tissues involved in cancer growth and metastasis, and devices capable of predicting molecular changes to prevent action against precancerous cells. Nanomaterials-based delivery systems in Theranostics (Diagnostics & Therapy) provide better penetration of therapeutic and diagnostic substances within the body at a reduced risk in comparison to conventional therapies. At the present time, there is a growing need to enhance the capability of theranostics procedures where nanomaterials-based sensors may provide for the simultaneous detection of several gene-associated conditions and nanodevices with the ability to monitor real-time drug action. These innovative multifunctional nanocarriers for cancer theranostics may allow the development of diagnostics systems such as colorimetric and immunoassays, and in therapy approaches through gene therapy, drug delivery and tumor targeting systems in cancer.

Some of the thousands and thousands of published nanosystems so far will most likely revolutionize our understanding of biological mechanisms and push forward the clinical practice through their integration in future diagnostics platforms. Nevertheless, despite the significant efforts towards the use of nanomaterials in biologically relevant research, more in vivo studies are needed to assess the applicability of these materials as delivery agents. In fact, only a few went through feasible clinical trials. Nanomaterials have to serve as the norm rather than an exception in the future conventional cancer treatments. Future in vivo work will need to carefully consider the correct choice of chemical modifications to incorporate into the multifunctional nanocarriers to avoid activation off-target, side effects and toxicity. Moreover the majority of studies on nanomaterials do not consider the final application to guide the design of nanomaterial. Instead, the focus is predominantly on engineering materials with specific physical or chemical properties.

It is imperative to learn how advances in nanosystem's capabilities are being used to identify new diagnostic and therapy tools driving the development of personalized medicine in oncology; discover how integrating cancer research and nanotechnology modeling can help patient diagnosis and treatment; recognize how to translate nanotheranostics data into an actionable clinical strategy; discuss with industry leaders how nanotheranostics is evolving and what the impact is on current research efforts; and last but not least, learn what approaches are proving fruitful in turning promising clinical data into treatment realities.

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EDITORIAL
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Editorial: Cancer Nanotheranostics: What Have We Learned So Far?

João Conde[1,2], Furong Tian[3], Jesus M. de la Fuente[4] and Pedro V. Baptista[5]*

[1] Harvard-MIT Division for Health Sciences and Technology, Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA, USA, [2] School of Engineering and Materials Science, Queen Mary University of London, London, UK, [3] School of Food Science and Environmental Health, College of Sciences and Health, Dublin Institute of Technology, Dublin, Ireland, [4] Instituto de Ciencia de Materiales de Aragón, Consejo Superior de Investigaciones Científicas/Universidad de Zaragoza, Zaragoza, Spain, [5] Research Unit on Applied Molecular Biosciences (UCIBIO), Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Lisboa, Portugal

Keywords: oncology, cancer, nanotechnology, nanotheranostics, nanomaterials, nanoparticles

The Editorial on the Research Topic

Cancer Nanotheranostics: What Have We Learned So Far?

According to the *National Cancer Institute*, in 2015 an estimated of 1.7 million new cases of cancer will be diagnosed only in the United States and around 600,000 people will die from the disease. The most common type of cancer is breast cancer, with more than 234,000 new cases expected in the United States in 2015. The next most common cancers are prostate cancer and lung cancer.

After a quarter of century of rapid technological advances, research has revealed the complexity of cancer, a disease intimately related to the dynamic transformation of the genome. These transformations trigger a range of modification to cell processes and molecular events that initiate and promote tumor genesis and progression, then local invasion and metastasis, i.e., the hallmarks of cancer development. These alterations may cause a wide scope of "diseases" that share similar molecular patterns that cause transformation and malignancy. Each of this stepwise evolution of the initial molecular event drives abnormal growth and loss of differentiation that ultimately causes tissue and organ failure. The initial molecular event may lay within the erroneous expression of a given gene, epigenetic modification and/or sporadic mutations occurring on genomic DNA during the life span of organisms. Each and every one of these molecular events may be evaluated and used as diagnostics biomarker and therapeutic target. For example, therapy action may target a mutated gene and silence its expression so as to avoid erroneous protein expression that mutates cell function. However, the full understanding of the molecular onset of this disease is still far from achieved and the search for mechanisms of treatment will follow closely.

It is here that Nanotechnology enters the fray offering a wealth of tools to diagnose and treat cancer. Today, Nanotechnology is a burgeoning field that is helping to address critical global problems from cancer treatment to climate change. In fact, Nanotechnology is everywhere and is everyday practice (Conde), offering numerous tools to diagnose and treat cancer, such as new imaging agents, multifunctional devices capable of overcome biological barriers to deliver therapeutic agents directly to cells and tissues involved in cancer growth and metastasis, and devices capable of predicting molecular changes to prevent action against precancerous cells (Conde et al., 2012). The novel physical properties of inorganic particles at the nanometer size scale, combined with the high specific surface of polymeric nanoparticles and the possibility to engineer stimuli-respond drug release strategies, have provided new tools to physicians for the diagnostic and the therapy of diseases such as cancer.

Nanomaterials-based delivery systems in Theranostics (Diagnostics and Therapy), at the same size level of proteins, DNA or RNA, provide better penetration of therapeutic and diagnostic

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Edited and reviewed by:
Gil Garnier,
Bioresource Processing Research Institute of Australia, Australia

****Correspondence:***
João Conde
jdconde@mit.edu;
conde.bio@gmail.com

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substances within the body at a reduced risk in comparison to conventional therapies. At the present time, there is a growing need to enhance the capability of theranostics procedures where innovative multifunctional nanocarriers for cancer theranostics may allow the development of diagnostics systems such as colorimetric and immunoassays, and in therapy approaches through gene therapy, drug delivery and tumor targeting systems in cancer (Conde et al., 2014).

Some of the thousands and thousands of published nanosystems so far will most likely revolutionize our understanding of biological mechanisms and push forward the clinical practice through their integration in future diagnostics platforms. Nevertheless, despite the significant efforts toward the use of nanomaterials in biologically relevant research, more *in vivo* studies are needed to assess the applicability of these materials as delivery agents. In fact, only a few have gone through feasible clinical trials. Nanomaterials have to serve as the norm rather than an exception in the future conventional cancer treatments. Future *in vivo* work will need to carefully consider the correct choice of chemical modifications to incorporate into the multifunctional nanocarriers to avoid activation off-target, side effects and toxicity.

It is imperious to learn how advances in nanosystems' capabilities are being used to identify new diagnostic and therapy apparatuses driving the development of personalized and precision medicine in cancer therapy and diagnostics; learn how incorporating cancer research and nanotechnology can help patient life quality; identify how to decipher nanotheranostics data into a real clinical strategy; and, last but not least, learn what methods are showing fertile results in turning promising clinical data into treatment realities (Conde et al., 2014).

Although all studies described in this Topic provide a baseline level of data in support of the effectiveness and safety of nanomaterials, we wonder what have we learned so far?

Current trends in biomedicine have been focused toward the use of new materials capable to address particular and individual characteristics in strategies for molecular precision therapies. In this endeavor, nanoparticles have allowed a tremendous leap forward in combining diagnostics and therapy in a single system and doing so at the nanoscale. Nanotheranostics have enabled the integration of targeting, imaging and therapeutics in a single platform, with proven applicability on the management of heterogeneous diseases.

Despite the plethora of proposed systems, only but a few products are currently included clinical trials and much remains to be done to allow effective clinical translation of these promising nanotheranostics platforms.

Several nanoconjugates have been proposed, varying in material, size and shape; some bringing current therapeutic approaches to the nanometer scale while others enact disruptive properties only possible by combination of different molecules and chemistries at the nanoscale (Conde et al.). For example, achieving controlled cellular responses of nanoparticles is critical for the successful development and translation of NP-based drug delivery systems. Conde et al. and Hong et al. (Pearson et al.) reported a complete survey on the most important factors for careful design of nanoparticles and the demand for precise control over the physicochemical and biological properties of NPs.

Liu et al. discuss the potential of star shaped nanoparticles in novel imaging approaches and strategies combining therapy and imaging in cancer. In fact, the potential of application of nanoconjugates in enhanced imaging strategies and platforms is discussed by Alcantara et al. with particular emphasis in current trends in molecular imaging for optimized management of breast cancer.

Theranostics of brain diseases such as brain cancer, is a daunting challenge due to the unique environment of central nervous system (Bhaskar et al., 2010). Yet passing the blood-brain barrier (BBB) is particularly difficult. The proper design of such engineered "nanocarriers" becomes very important in translocating the impermeable membranes of the brain to facilitate drug delivery. At the same time, it is also required to retain the drug stability and ensure that early degradation of drugs from the nanocarriers does not take place. In fact, Mahmoudi and Hadjipanayis reported a great opinion piece about the application of magnetic nanoparticles (MNPS) for the treatment of brain tumors and how MNPs will likely assume a larger role in brain cancer treatment in combination with other adjuvant therapies.

Talking about other adjuvant therapies, radiation and gene therapy have also gained momentum in the last years when using nanomaterials for cancer therapy. Cooper et al. reported how radiation therapy is one of the most commonly used treatments for cancer and which directions to follow for the future based on current state of nanoparticle-assisted radiation therapy.

Regarding gene therapy, Moreno and Pego reported a critical overview of using therapeutic antisense oligonucleotides against cancer and how difficult has been to get to the clinic. This is in fact not only a problem with gene therapy but a universal issue as whilst many pre-clinical data has been generated, a lack of understanding still exists on how to efficiently tackle all the different challenges presented for cancer targeting in a clinical setting.

Perhaps another interesting avenue in cancer nanotheranostics is the interfering effect of the immune system in the efficacy of proposed platforms. In fact, a clear perspective on the interaction between immune response and immune modulators is still missing from the general picture of nanotheranostics, not only in what concerns the organisms response to the systemic delivery of nanoconjugates that may hamper efficacy, but also the use of the immune response and nanoconjugates interaction with immune system as means to achieve higher and more directed/targeted therapy to the cancer site. As such, the effect and response of diverse properties of nanodiagnostics platforms in the organisms have been discussed by Clift et al. where nanoconjugates are discussed in terms of the immune response triggered after systemic delivery; whereas Conniot et al. and Pearson et al. (Dawidczyk et al.) have demonstrated how nanotheranostics may use and profit from the specific and unspecific immune response to enhance efficacy. Actually, cancer immunotherapy is nowadays consider a hot topic and a huge breakthrough in modern Science (Conde et al., 2015).

Gene expression has been targeted for silencing to avoid mutated protein function to exert its role in tumor progression. Nanotechnology based systems shows great promise in addressing novel genomic biomarkers that signal cancer cells, and do it with increased sensitivity that allow early detection of genome/genetic modifications that at the origin of cancer. Emergent technologies have been combined with nanoscale structures for directing to the site of interest with decreased side effects. The experience gathered thus far has shown that the next step in the effective translation of nanotheranostics into the clinics relates to the body's response to the nanoconjugates. What are the toxicity impacts of these devices and platforms? Are there enough data for the full chronic toxicity evaluation of the application of these systems? Is the immune system a friend or foe for nanotheranostics?

AUTHOR CONTRIBUTIONS

All the authors contributed to this Editorial piece. All the authors read and revised the manuscript.

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Revisiting 30 years of biofunctionalization and surface chemistry of inorganic nanoparticles for nanomedicine

João Conde[1]*†, Jorge T. Dias[2†], Valeria Grazú[2]*, Maria Moros[2], Pedro V. Baptista[3] and Jesus M. de la Fuente[2,4,5]

[1] *Harvard-MIT Division for Health Sciences and Technology, Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA, USA*
[2] *Nanotherapy and Nanodiagnostics Group, Instituto de Nanociencia de Aragon, Universidad de Zaragoza, Zaragoza, Spain*
[3] *CIGMH, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal*
[4] *Fundacion ARAID, Zaragoza, Spain*
[5] *Key Laboratory for Thin Film and Microfabrication Technology of the Ministry of Education, Department of Bio-Nano Science and Engineering, Institute of Nano Biomedicine and Engineering, Research Institute of Translation Medicine, Shanghai Jiao Tong University, Shanghai, China*

Edited by:
Jean-Michel Lavoie, Université de Sherbrooke, Canada

Reviewed by:
Victor Sans Sangorrin, University of Glasgow, UK
Leonie Rouleau, Université de Sherbrooke, Canada

****Correspondence:***
João Conde, Harvard-MIT Division for Health Sciences and Technology, Institute for Medical Engineering and Science, Massachusetts Institute of Technology, 45 Carleton Street, E25-438, Cambridge, MA 02139, USA
e-mail: jdconde@mit.edu;
Valeria Grazú, Nanotherapy and Nanodiagnostics Group, Instituto de Nanociencia de Aragon, Universidad de Zaragoza,Campus Rio Ebro, Edifício ID, Mariano Esquillor s/n, 50018 Zaragoza, Spain
e-mail: vgrazu@unizar.es

† These authors have contributed equally to this work.

In the last 30 years we have assisted to a massive advance of nanomaterials in material science. Nanomaterials and structures, in addition to their small size, have properties that differ from those of larger bulk materials, making them ideal for a host of novel applications. The spread of nanotechnology in the last years has been due to the improvement of synthesis and characterization methods on the nanoscale, a field rich in new physical phenomena and synthetic opportunities. In fact, the development of functional nanoparticles has progressed exponentially over the past two decades. This work aims to extensively review 30 years of different strategies of surface modification and functionalization of noble metal (gold) nanoparticles, magnetic nanocrystals and semiconductor nanoparticles, such as quantum dots. The aim of this review is not only to provide in-depth insights into the different biofunctionalization and characterization methods, but also to give an overview of possibilities and limitations of the available nanoparticles.

Keywords: biofunctionalization, chemistry surface, gold nanoparticles, magnetic nanoparticles, quantum dots

INTRODUCTION

Every object with at least one characteristic dimension between 1 and 100 nm can be defined as "nanomaterial." The importance of nanomaterials (e.g. nanoparticles, NPs) for science and technology has highly increased in the last years (Surendiran et al., 2009). When dealing with such a small structure, the size-related properties, the shape and inter-particle distance to the core, the charge, the dielectric properties of the conjugated system (including refractive index and polarizability), the dielectric medium surrounding the particle (solvent) and the composition moieties are extremely important and may sturdily influence the physical and chemical characteristics of the nanomaterials. This means that these distinct properties, such as quantum confinement in semiconductor nanocrystals or surface plasmon resonance (SPR) in some metal NPs, may influence physical and chemical behavior of nanomaterials (Bellucci, 2009; Doria et al., 2012).

The unbelievable development of nanotechnology in the last 30 years has allowed the release of new and efficient synthetic routes toward the production and functionalization of different NPs, composed of a variety of materials including noble metals [e.g. gold and silver (Conde et al., 2012c; Doria et al., 2012; Dreaden et al., 2012)], semiconductors [e.g. CdSe and CdTe (Murray et al., 1993), TiO_2 (Sudhagar et al., 2012), InP (Xu et al., 2006)], magnetic compounds (Pankhurst et al., 2003), and their combinations, such as core–shell (Cao et al., 2001) and alloy NPs (Doria et al., 2010).

The unique characteristics of these NPs, such as high surface-to-volume ratio or size-dependent optical and magnetic properties, are drastically different from those of their bulk materials and hold pledge in the clinical field (Kim, 2007; Heath and Davis, 2008). Technological advances in nanoparticle synthesis/functionalization are producing significant advances in molecular detection and imaging, target and multifunctional therapeutics and in prevention/control of diseases. Through the development of new imaging agents, novel multifunctional targeted devices capable of overcoming biological barriers for

direct delivery of therapeutic agents to diseased cells and tissues, and innovative monitoring sensors for predictive molecular changes, increase the processes' efficiency while minimizing costs (Baptista, 2009; Minelli et al., 2010; Ma et al., 2011; Conde et al., 2012c). Besides being an area of intense upfront basic research, nanotechnology holds the key to future technological applications. It is a burgeoning field as more and improved techniques are becoming available for clinical therapy and diagnostics with increased sensitivity and efficiency at lower costs (Baptista et al., 2008; Lammers et al., 2011).

In spite of these advantages, NPs do have limitations. For example, their small size and large surface area can lead to particle-particle aggregation and may result in limited loading of functional components and burst release. In fact, only NPs with the appropriate size and surface chemistry are not immediately recognized by the immune system and show increased circulation times (Gil and Parak, 2008; Shvedova et al., 2010). Nevertheless, their unique and broad-based optical properties, their ease of synthesis and facile surface chemistry and, most importantly, their appropriate size scale are overcoming these drawbacks and have been generating much eagerness in clinical diagnostics and therapy (Sperling et al., 2008).

Here we will review different strategies of biofunctionalization and characterization methods of inorganic nanoparticles, as well as their main advantages and limitations. Our aim is to discuss the challenges of working with nanoparticles while giving an overall overview of the state-of-the-art.

INORGANIC NANOPARTICLES

GOLD NANOPARTICLES

Historically, colloidal gold has been used since ancient times mainly as a method for glass staining. However, it was not until Faraday's work that gold nanoparticles (AuNPs) began to be studied in a scientific approach (Faraday, 1857). Since then, AuNPs have generated ever-increasing interest and in the last few decades more and more controllable synthesis methods and applications in diverse nanosystems have been developed (Wagner et al., 2000; Edwards and Thomas, 2007).

AuNPs, also known as colloidal gold, are a suspension of sub-micrometer-sized gold metal particle in a fluid and can be obtained with diameters between 3 and 200 nm. AuNPs have gained increasing interest due to their special features, such as extraordinary optical and electronic properties, high stability and biological compatibility, controllable morphology and size dispersion, and easy surface functionalization (Sperling et al., 2008).

The optical properties of AuNPs are significant because absorption and emission are within the visible range of light (El-Sayed, 2001). Of particular interest is the light extinction process in the UV-visible range, which occurs when an electromagnetic wave passes through a metal particle exciting its electronic or vibrational states (Kreibig and Vollmer, 1995). This phenomenon induces dipole moments that oscillate at the respective frequency of the incident wave, dispersing secondary radiation in all directions. This collective oscillation of the free conduction electrons is called localized surface plasmon resonance (LSPR). The SPR is the collective oscillation of the electrons in the conduction band. The oscillation frequency is usually in the visible region giving rise to the strong SPR absorption (Schultz et al., 2000; Jain et al., 2006; Huang et al., 2007; Murphy et al., 2008).

Size also provides important control over many of the physical and chemical properties, including luminescence, conductivity, and catalytic activity. AuNPs' absorption and scattering proportions depend on the AuNPs size (Cao et al., 2002). AuNPs with a diameter smaller than 20 nm essentially show absorption, but when the size increase to 80 nm the ratio of scattering to absorption also increases. As the size of the AuNPs increases, light can no longer polarize the nanoparticles homogeneously and higher order modes at lower energy dominate. This causes a red-shift and broadening of the surface plasmon band. Small AuNPs, like those with 13 nm of diameter, absorb green light, which corresponds to a strong absorption band at 520 nm in the visible light spectrum. However, solutions of AuNPs appear red in color. For smaller AuNPs (i.e., 5 nm diameter), surface electrons are oscillated by the incoming light in a dipole mode, but the SPR is very sensitive to the composition, size, shape, inter-particle distance and environment (dielectric properties) of the AuNPs (Pellegrino et al., 2005; Sperling et al., 2006).

In all synthesis methods reported so far, a reducing and a stabilizing agent is added to prevent the particles from aggregating. The type of stabilizer affects the selection of further biofunctionalization strategies, as for certain strategies a good colloidal stability at a broad range of pH and ionic strength is required. Although a wide variety of nanoparticles can be stabilized by a large range of stabilizers (ligands, surfactants, polymers, dendrimers, biomolecules) (Sperling and Parak, 2010), the most robust nanoparticles are covered by thiol molecules using the strong gold–S bond between the soft acid Au and the soft thiolate base (Giersig and Mulvaney, 1993). Thus, if a bifunctional thiolated stabilizer or even a mixture of them is used (e.g. thiolated-PEG molecules having carboxylic, amine, azide groups, etc.), then it is possible to stabilize and introduce chemical functionalities in a single step. This allows further attachment of biomolecules in a controlled way by covalent immobilization strategies that we will discuss in the following sections.

MAGNETIC NANOPARTICLES

Magnetic nanoparticles (MNPs) constitute an important class of nanomaterials widely studied for their potential use in biomedicine fields, such as imaging, cell labeling, hyperthermia and drug and gene delivery (Pankhurst et al., 2003; Barakat, 2009; Berry, 2009). MNPs can be classified as metal, alloys or oxides, and are generally based on elements such as iron, cobalt, nickel, or manganese among others (Pankhurst et al., 2009). From the aforementioned, iron-based NPs are the most studied, since iron is believed to be biocompatible (Hanini et al., 2011). Other elements, such as cobalt or nickel are reported to be more toxic (Fang and Zhang, 2009). Iron oxide NPs (IONPs) are composed of magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3), nanocrystallites.

Most of MNPs' applications are a consequence of their magnetic properties, which greatly differ from those of the bulk material. When small enough, MNPs present superparamagnetic behavior at room temperature, meaning that they become magnetized upon exposure to an external magnetic field but lack remnant magnetization once the external field is removed (Jun

et al., 2008). Consequently, MNPs do not agglomerate in the absence of the magnetic field, which is essential for *in vivo* applications (Yoo et al., 2011). This characteristic is rather important in applications, such as magnetic hyperthermia. The MNPs' capacity of converting the energy of an alternate magnetic field into heat (Rosensweig, 2002) and the extra sensitiveness of tumor cells to an increase in temperature (van der Zee, 2002) are the two pillars of magnetic hyperthermia in cancer. Since the late 50's, when Gilchrist et al. (1957) first reported the use of MNPs to heat tissue samples, to nowadays, magnetic hyperthermia has evolved considerably and is a key area of interest in cancer therapy with several studies showing the benefit of employing magnetic materials in hyperthermia strategies (Jordan et al., 1993, 2001; Johannsen et al., 2010; Laurent et al., 2011). Several groups have reported noteworthy results in clinical trials where magnetic hyperthermia shows effectiveness in tumor cell destruction with impressive targeting, thus minimizing significantly side effects (Johannsen et al., 2005; Liu et al., 2011; Zhao et al., 2012b).

There are a wide variety of methodologies used for MNP synthesis, including physical or wet chemical approaches. Concerning wet chemical approaches, there are some methodologies, such as coprecipitation (Perez et al., 2002) or reverse micelles precipitation (Liu et al., 2000) that provide directly water soluble MNPs with an organic layer with chemical moieties for narrow size distribution of MNP. However, common synthetic strategies traditionally render MNPs soluble only in organic solvents. Their use in bioapplications imply an additional step where adequate chemical moieties are introduced by several strategies (e.g. use of amphiphilic polymers, silanization, replacing and/or modifying the surfactant layer) in order to allow silanization, their water transference and further biofunctionalization.

QUANTUM DOTS

Quantum dots (QDs) are nanoparticles composed of semiconductor materials from III-V or II-VI groups of the periodic table, such as ZnS, ZnSe, CdS, CdSe, CdTe, InP, and others (Donega, 2011). Their reduced size induces a shift of the electronic excitations to higher energy, concentrating the oscillator strength into just a few transitions, conferring unique quantum-confined photonic and electronic properties (Alivisatos, 1996; Alivisatos et al., 2005). Although physically larger than organic dyes and fluorescent proteins, their cumulative optical properties offer great biological utility. With tunable core sizes, it is possible to attain a broad adsorption profile, narrow size, and symmetric photoluminescence spectra depending of the fundamental materials. QDs also show strong resistance to photobleaching and chemical degradation, as well as significant photostability and high quantum yields (Ghanem et al., 2004; Xu et al., 2006; Algar et al., 2011).

Their potential as biological labels was first demonstrated by Nie and Alivisatos groups in 1998, turning the focus into bioapplications of QDs. The method relies on a ligand exchange strategy is based on the replacement of the original hydrophobic ligands adsorbed onto the surface of QDs with biofunctional molecules, such as protein transferrins. These QDs were susceptible to effective receptor-mediated endocytosis in cultured HeLa cells. Since these first demonstrations of QDs potential, their unique properties have been continuously optimized and applied in a plethora of bioapplications, ranging from fluorescent probes, biosensors to therapeutics and theranostic agents (Akerman et al., 2002; Smith et al., 2006; Li et al., 2009; Liu et al., 2010; Ruan et al., 2012; Singh et al., 2012).

Once QDs that show paramount optical properties are those synthesized in organic media, numerous methods have been developed for creating hydrophilic QDs (Medintz et al., 2008). The first approach is commonly designated as "ligand exchange" (Gill et al., 2008), where the hydrophobic layer of the organic solvent may be replaced by biofunctional molecules containing a soft acidic group (i.e., thiol, sodium thiolycolate) and hydrophilic groups (i.e., carboxylic, aminic groups) (Wang et al., 2008). A second approach usually consists in adding a particular shell to the nanoparticles that can be further functionalized with additional biomolecules or polymers (Koole et al., 2008; Zhang et al., 2008).

BIOFUNCTIONALIZATION OF INORGANIC NANOPARTICLES

Nanoparticles with unique and broad-based optical properties, ease of synthesis and facile surface chemistry and functionalization within appropriate size scale are generating much enthusiasm in biotechnology and biomedicine, with particular emphasis in clinical diagnostics and therapy. However, for the biological application of these NPs, it is necessary their functionalization with one or several biomolecule (**Figure 1**), such as DNA/RNA, oligonucleotides (i.e., ssDNA/RNA, dsDNA/RNA), peptides and antibodies, fluorescent dyes, polymers (i.e., PEGs), drugs, tumor

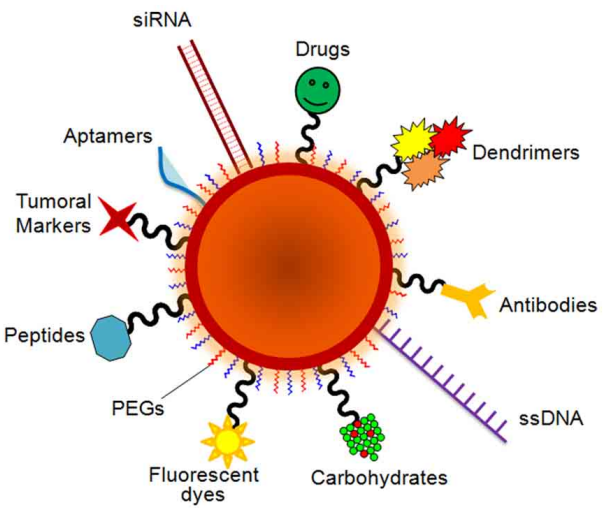

FIGURE 1 | Schematic representation of a multifunctional nanocarrier. These innovative NPs comprise nucleic acids such as RNA and DNA used for gene silencing approaches and in colorimetric assays, respectively. Aptamers and anticancer drug molecules are also used for delivery to the target tissue. Carbohydrates may be useful as sensitive colorimetric probes. PEG is used to improve solubility and decrease immunogenicity. Responsive nanocarriers can also trigger reaction upon external stimuli through the functionality of valuable tumor markers, peptides, carbohydrates, polymers and antibodies that can be used to improve nanocarrier circulation, effectiveness, and selectivity. Multifunctional systems can also carry fluorescent dyes that are used as reporter molecules tethered to the particle surface and employed as tracking and/or contrast agents.

markers, enzymes and other proteins that will introduce the required bio-functionalities.

Ultimately, the conjugation strategy is directly dependent on a numbers of factors such as size, surface chemistry and shape, as well as the type of ligands and functional groups to exploit in the functionalization. Also, the type of biological molecule and the final application of the nanoparticle conjugate are crucial when evaluating the conjugation strategy. Next, we summarize the most frequently used biofunctional molecules used to introduce one or several biological activities to the NP.

COMMON BIOFUNCTIONAL SPECIES

Polymer coatings—poly(ethylene glycol)

For their use as potential delivery devices *in vivo,* the aforementioned inorganic nanoparticles must have long plasma half-lives. In this sense, poly(ethylene glycol) (PEG) is the most widely used macromolecule to prolong nanocarriers half-life. In fact, PEGs have a strong effect on nanoparticle structure, stabilization and biodistribution both *in vitro* and *in vivo* (Akerman et al., 2002; Daou et al., 2009; Boeneman et al., 2010; Maldiney et al., 2011). These long-circulating nanoparticles have the ability to circulate for a prolonged period of time and target a particular organ, as carriers of DNA in gene therapy, or to deliver proteins, peptides and drugs (Langer, 2000; Bhadra et al., 2002; Kommareddy et al., 2005; Lee and Kim, 2005).

For systemic applications, the development of surface functionalized and long-circulating NPs as cellular probes and delivery agents is highly desired for passive targeting to tumors and inflammatory sites. PEG-modification of NPs affords long circulating property by evading macrophage-mediated uptake and removal from the systemic circulation. Owing to its simple structure and chemical stability, it is a prototype of an inert and biocompatible polymer (Sperling and Parak, 2010; Verma and Stellacci, 2010). When bound to surfaces, PEG prevents other molecules to bind by steric effects. In fact, the molecules are not attracted by electrostatic forces and cannot penetrate the hydrated PEG layer, producing an inert hydrophilic surface. Moreover, PEG modified nanoparticles are more stable at high salt concentrations and in biological environments, avoiding non-specific binding to proteins and cells (Sperling and Parak, 2010). This is particularly important for *in vivo* applications because once the NPs are in the bloodstream, a portion of the plasma proteins that can adsorb to the surface (opsonins), may promote NPs recognition by the mononuclear phagocyte system (MPS), and consequently lead to rapid removal of the NPs from circulation (Bertrand and Leroux, 2012). To date, there is a general consensus that to prolong NPs half-life in the organism, PEGs' molecular weight, grafting density and chain architecture must be optimized (Li and Huang, 2010; Grazú et al., 2012). For instance, Xie and coworkers showed that MNPs functionalized with PEG with molecular weights higher than 3000 Da were not taken up by macrophages *in vitro,* while extensive uptake was observed for PEG 600-coated MNPs (Xie et al., 2007).

Consequently, functionalization of NPs with a high density of PEG of an adequate length not only increases the colloidal stability of the modified NPs but also their plasma half-life. However, to provide PEGylated NPs with targeting and therapeutic activity, as well as with the ability of crossing different biological membranes, they must be conjugated with a variety of biologically relevant ligands, such as cell/tumor penetrating peptides, tumor markers, and therapeutic agents (siRNAs, drugs). Concerning gold NPs, one of the main strategies is to assemble PEG and mixed biomolecule/PEG monolayers on the nanoparticles' surface. Liu et al. showed an escalation in the NPs' stability with increasing PEG length, decreasing nanoparticle diameter, increasing PEG mole fraction and mixed monolayers prepared via the sequential addition of PEG followed by a peptide. In this manner, NPs were more stable than those prepared via simultaneous co-adsorption. These modified NPs were able to target the cytoplasm of HeLa cells, being the cellular uptake quantified using inductively coupled plasma optical emission spectrometry (Liu et al., 2007). Sanz et al. also obtained polyvalent PEGylated AuNPs with a similar strategy. The authors developed an approach to attach specific biomolecules to the AuNPs' surface and their effect in the functionalization with other specific derivatives. The effect of biofunctional spacers, such as thiolated PEG chains and a positive peptide (TAT) in dsRNA loading on AuNPs was reported. The authors hypothesized that the loading of oligonucleotides onto the AuNP surface may be controlled by ionic and weak interactions positioning the entry of the oligonucleotide through the PEG layer, by a synergistic effect of the TAT peptide and PEG chains with specific functional groups, enhancing the dsRNA loading onto AuNPs (see **Figure 2**) (Sanz et al., 2012).

Another approach to link biomolecules to PEGylated AuNPs is making use of PEG as a spacer. This requires the use of bifunctional PEG chains that contain thiol at one end and a suitable functional moiety at the other (e.g. amino, carboxylate groups). Recently, Oh et al. described a different approach, where in a one-phase synthesis AuNPs were conjugated with PEG ligands yielding a narrow size distribution of highly stable NPs in the presence of high salt concentrations over a wide range of pHs (Oh et al., 2010b). One way or another, functional moieties of PEG ligands allow for further coupling of target biomolecules. Consequently, surface modification of gold clusters through PEG spacers (Kanaras et al., 2002; Simpson et al., 2011) would allow the modified nanoparticles to remain in the systemic circulation for prolonged periods and provide flexibility for efficient interaction with a target. Besides, using a combination of different bifunctional PEG spacers, gold nano-platforms can be multifunctionalized with a variety of biologically-relevant ligands such as cell penetrating peptides, fluorescent dyes, tumor markers and siRNA (Conde et al., 2012a).

PEGylated QDs have also been successfully produced for effective *in vitro* and *in vivo* circulation (Skaff and Emrick, 2003; Hu et al., 2010; Prow et al., 2012; Yang et al., 2012b). Recently, Poulose et al. developed highly biocompatible PEG functionalized in cadmium chalcogenide luminescent QDs (CdS, CdSe, and CdTe) as an imaging tool for early diagnosis of cancer by targeting a cancer cell line (Poulose et al., 2012).

Although PEG is really useful to prolong NPs' blood half-life, it is known that in some cases PEG can hamper cargo release or hide other functional domains once the NP accumulates at the desired target area (Sawant and Torchilin, 2012). Inclusion of

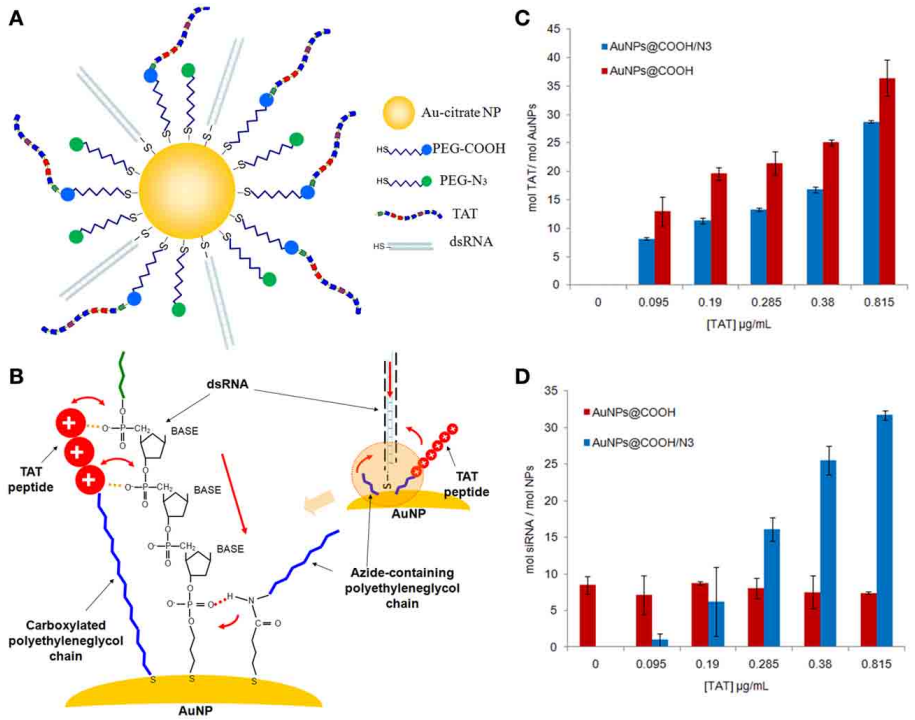

FIGURE 2 | Polyvalent PEGylated gold nanoparticles. (A) Bioconjugation of the surface-modified gold nanoparticles with different thiol-PEG layer composition (SH-EG(7)-CH_2-COOH and SH-$(CH_2)_3$-CONH-EG(6)-CH_2-N_3), TAT peptide and thiol-dsRNA oligonucleotide. **(B)** Mechanism for the enhancement of the dsRNA loading on AuNPs functionalized with PEG chains and TAT peptide. The azide group has a resonant structure with a positively polarized behavior that can attach the negatively charged thiolated oligonucleotide to the gold surface. The azide-containing chain also encloses an amide group near the gold surface that could play a role in approaching the thiol group of the oligonucleotide to the gold surface. This amide group could form a hydrogen bond with one of the hydroxyl groups of the ribose group near the thiol group on the oligonucleotide. **(C)** Determination of the number of TAT chains bound to AuNPs by the EDC reaction as a function of the initial peptide concentration in the reaction mixture. Blue bars AuNP@COOH/N_3 and red bars AuNP@COOH. **(D)** Loading of thiolated oligonucleotide (HS-dsRNA) on AuNPs functionalized with TAT peptide and with both PEG-azide and PEG-COOH and only with PEG-COOH. Blue bars AuNP@COOH/N_3 and red bars AuNP@COOH (Sanz et al., 2012). Reproduced with permission from Sanz et al. (2012), Copyright 2013.

stimulus-sensitive detachable PEG is a possible solution to overcome these drawbacks, so that cargoes can be released or other ligands unveiled in response to microenvironmental conditions. For instance, Harris et al. functionalized MNPs with a PEG tethered by an MMP-2 cleavable substrate (Harris et al., 2008), being MMP-2 a protease upregulated in angiogenesis and metastasis. Once NPs reached the tumor, the polymer was cleaved, unveiling the cell penetrating peptide, resulting in increased uptake by cells when compared to non-cleavable PEG.

Fluorescent dyes

Several studies report on the modulation of fluorophores at the vicinity of nanoparticles (Kang et al., 2011; Rosa et al., 2011), which has found application in a variety of systems to detect biologically relevant targets.

Several methods based on the quenching of fluorescence have been proposed for DNA detection consisting of fluorophore-labeled ssDNA electrostatically adsorbed onto AuNPs (Ray et al., 2006), where the presence of a complementary target triggers desorption of the newly formed dsDNA from the nanostructures due to the electrostatic variation between ssDNA and dsDNA, and fluorescence emission is restored. Also, fluorescence quenching of fluorophores close to gold nanocarriers functionalized with thiol-modified oligonucleotides has been explored in different conformations (Wu et al., 2006; Tang et al., 2008). Tang et al. proposed a method to probe hydroxyl radicals using an AuNP-oligonucleotide-FAM system where the hydroxyl radical promotes strand breakage and consequent release of FAM, restoring the previously quenched fluorescence (see **Figure 3**) (Tang et al., 2008). The same quenching mechanism was used to detect specific DNA strands using two probes (one with an AuNP label and another labeled with TAMRA) that hybridize to two DNA sequences near each other (Wu et al., 2006), bringing the fluorophore and AuNP close enough to quench fluorescence emission.

Proteins have also been probed through nanoparticle fluorescence-mediated systems, especially for protein detection via quenching, through the interaction with fluorescent AuNPs (Mayilo et al., 2009; He et al., 2010; Lacerda et al., 2010). Due to their efficient proximity-dependent fluorescence quenching they can be used *per se* or as part of more elaborate conjugates (i.e., with QDs) (Pons et al., 2007). One example is the work of De et al. where the interaction between AuNPs and the green fluorescent protein (GFP) was employed to detect proteins in

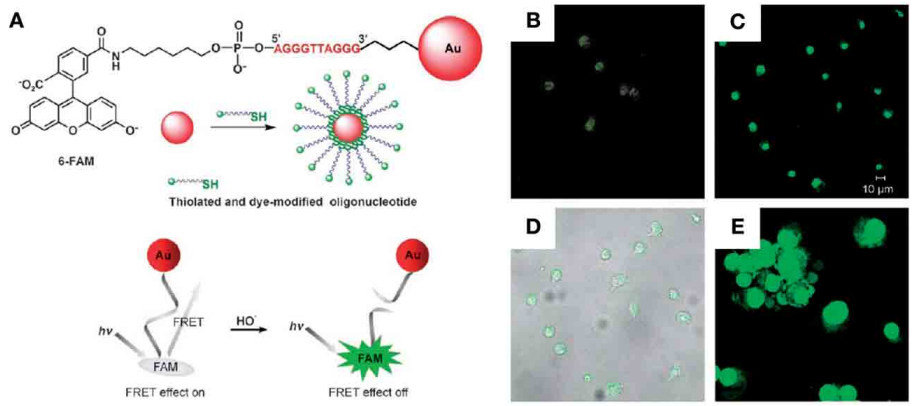

FIGURE 3 | Fluorescent-AuNPs. (A) Chemical structure of a FAM–DNA–AuNP and schematic illustration of its FRET-based operating principles. **(B–E)** Confocal fluorescence and phase-contrast images of living cells. **(B)** Fluorescence image of macrophages incubated with the probe for 30 min at 37°C. **(C)** Fluorescence image of probe-stained macrophages stimulated with PMA for 1 h at 37°C. **(D)** Bright field image of live macrophages shown in **(C)**, confirming their viability. **(E)** AO staining of probe-loaded macrophages, confirming their viability (Tang et al., 2008). Reproduced with permission from Tang et al. (2008), Copyright 2013.

biological matrices, such as serum (De et al., 2009). By correlating the variation in fluorescence intensity with specific proteins of interest, they were able to identify proteins such as fibrinogen, human serum albumin and immunoglobin G, among others with over 97% accuracy.

In essence, fluorescent-nanoparticle systems can be used for sensing by exploring a typical FRET in order to provide efficient *in vivo* detection and tumor targeting. These nanocarriers symbolize an important class of materials with unique features suitable for biomedical imaging applications such as increased sensitivity in detection, high quantum yields for fluorescence and a bounty of novel applications in optics and nanophotonics for molecular diagnostics (Conde et al., 2012d).

QDs are often used as fluorescent molecules *per se*, since they are semiconductor nanoparticles with narrow, tunable, symmetrical emission spectra and high quantum yields (Weller, 1993; Bruchez et al., 1998). These characteristics were evidenced by Wu et al. using QDs modified with different cellular antigens enabling the simultaneous detection of two different targets in the same cell (Wu et al., 2003). It was also shown their higher brightness and photobleaching resistance when compared to organic dyes. These properties make QDs exceptional substitutes as fluorescence labels (Xing et al., 2006; Smith et al., 2010; Smith and Nie, 2012).

The inclusion of dyes onto MNPs allows the creation of multifunctional NPs, which might be used for MRI and optical imaging. These dual MNPs allow for multimodal imaging, which implies that the limitations of one imaging modality could be compensated by the other, creating a complementary effect (Louie, 2010). For instance, Medarova and co-workers reported the synthesis of a multifunctional MNP that included near-infrared optical imaging dye, peptides for membrane translocation and synthetic siRNA targeting a specific gene (Medarova et al., 2007). *In vivo* accumulation of the MNPs was assessed by MRI and optical imaging and the silencing efficiency was also probed by *in vivo* optical imaging.

Nucleic acids

Watson and Crick first described DNA as two helical chains each coiled around the same axis, consisting of simple and repeating units called nucleotides with backbones made of sugars and phosphate groups joined by ester bonds that run in opposite directions to each other. The importance of this molecule within living cells is undisputable (Watson and Crick, 1953). Besides their biological function, nucleic acids can be employed as polymeric molecules which will bind specifically to targets thanks to Watson–Crick base pairing (Fichou and Ferec, 2006).

Mirkin et al. (1996) described the use of a cross-linking method that relies on the detection of single-stranded oligonucleotide targets using two different gold nanoprobes, each of them functionalized with a DNA-oligonucleotide complementary to one half of the given target. This functionalization was achieved using the strong affinity of thiol or disulfide groups to the gold surface of the NPs, forming quasi-covalent bonds. By modifying a nucleic acid molecule with a thiol group in either the 5′ or the 3′ end it is possible to fine-tune the DNA assembly into the gold surface (Hurst et al., 2006), controlling variables such as salt concentration, oligo/NP ratio or nanoparticle size. This phenomenon indicates the potential of AuNPs modified with DNAs to be applied in biosensing or as DNA probes for diagnosis (Cao et al., 2005). These assays became an important mark in detection once they have PCR-like sensitivity, selectivity for target sequences, capacity for massive multiplexing, and most importantly, have the ability to be performed at the point of care.

Using a fluorescence-based method, Demers et al., have determined the number of thiol-derivatized single-stranded oligonucleotides bound to AuNPs and their extent of hybridization with complementary oligonucleotides in solution (Demers et al., 2000). Also, using a fluorescence method, Conde et al. reported the potential of a single molecular nanoconjugate to intersect all RNA pathways: from gene specific downregulation to silencing the silencers, i.e., siRNA and miRNA pathways, by using gold nanobeacons (**Figure 4**). These nanoconjugates functionalized

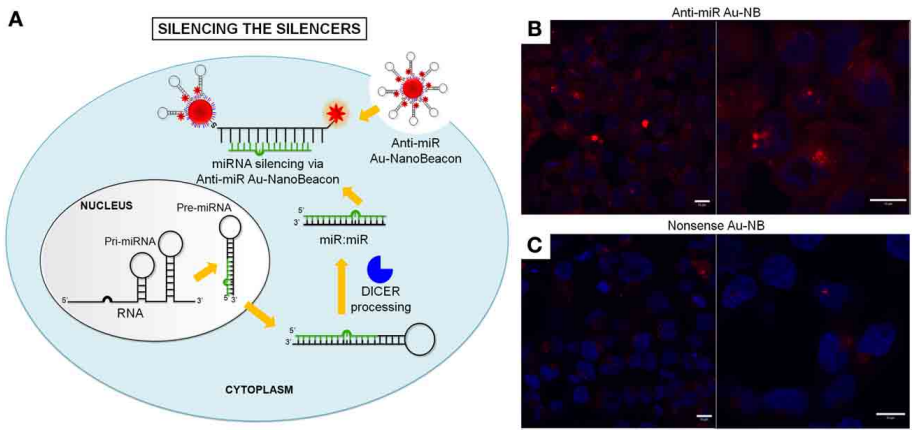

FIGURE 4 | Silencing the Silencers with hairpin-DNA-AuNPs—Gold nanobeacons. (A) Specific Au-nanobeacons are capable of intersecting miRNA pathway, leading to recovery of previously downregulated gene expression while simultaneously discriminating cells where silencing is occurring. The fluorescence signal may allow for tracking cell internalization and sub-cellular localization. The Au-nanobeacons' potential for anti-cancer therapeutics via the silencing of the silencers is demonstrated by blocking the endogenous microRNA pathway via an Anti-miR Au-nanobeacon complementary to the mature microRNA-21 (miR-21), commonly upregulated in cancer phenotypes. **(B,C)** Au-nanobeacons silencing of endogenous silencers—silencing of miR-21. Confocal imaging (scale bar, 10 μm) shows internalization of 50 nM **(B)** Anti-miR Au-nanobeacon 50 nM and **(C)** Nonsense Au-nanobeacon. Target (mature miR-21) recognition leads to change of Anti-miR Au-nanobeacon conformation in the cytoplasm with concomitant fluorescence signal (red, Cy3) encircling the cell nuclei (blue, DAPI) (Conde et al., 2013b). Reproduced with permission from Conde et al. (2013b), Copyright 2013.

with a fluorophore labeled hairpin-DNA are capable of efficiently silencing single gene expression, exogenous siRNA and an endogenous miRNA while yielding a quantifiable fluorescence signal directly proportional to the level of silencing. Inhibition of gene expression was achieved with concomitant increase of the gold nanobeacons' fluorescence that can be used to assess the silencing effect (Rosa et al., 2012; Conde et al., 2013a,b, 2014b).

Moreover, AuNPs functionalized with ssDNA are also capable of specifically hybridizing to a complementary target for the detection of a particular nucleic acid sequence in biological samples (Sato et al., 2003; Storhoff et al., 2004; Baptista et al., 2005; Thaxton et al., 2006). The impact of advances in these nanoparticle-based assays for specific detection of bioanalytes of clinical interest is particularly relevant in biodiagnostics, making them ideal candidates for developing biomarker platforms.

However, nucleic acid molecules are also capable of establishing ionic interaction in gold surface. It has been shown that both thiol-ssDNA and dsDNA stabilize gold nanoparticle dispersions, but possible non-specific interactions between the hydrophobic DNA bases and the gold surface promote interparticle interactions and cause aggregation within a short period of time (Cardenas et al., 2006). The charge repulsion among DNA strands and between DNA and AuNPs can be reduced by adding salt, reducing pH or by using non-charged peptide nucleic acid (PNA) (Zhang et al., 2012b).

Moreover, Conde et al. reported the design of two approaches (see **Figure 5**) for the binding of siRNA molecules to multifunctional AuNPs: (**Figure 5A**) the binding of the negatively charged siRNA through ionic interactions to the modified gold surface (ionic approach) and (**Figure 5B**) the use of thiolated siRNA for the binding to the nanoparticle through the strong interaction gold-thiol (covalent approach) (Conde et al., 2012a). The covalent approach was evaluated in a lung cancer mouse model to evaluate the inflammatory response and therapeutic siRNA silencing via RGD-nanoparticles. This study reported the use of siRNA/RGD gold nanoparticles capable of targeting tumor cells in two lung cancer xenograft mouse models, resulting in successful and significant *c-Myc* oncogene downregulation followed by tumor growth inhibition and prolonged survival of the animals (Conde et al., 2013c).

Modulation of the physicochemical properties of the gold nanocarriers can be easily achieved by adequate synthetic strategies and give AuNPs advantages over conventional detection methods currently used in clinical diagnostics and therapy. Simple and inexpensive methods based on these bio-nanoprobes were initially applied for detection of specific DNA sequences and are currently being expanded to clinical diagnosis (Baptista et al., 2008).

QDs have also been functionalized with nucleic acids and extensively used as DNA sensors (Crut et al., 2005; Dubertret, 2005; Zhang et al., 2005a; Choi et al., 2009; Ye et al., 2009; Zhang and Hu, 2010). For *in vitro* test of target DNA, QDs based FRET pairs turned out to be of great use. The method developed by Zhang et al. using QDs-Cy5-labeled reporter oligonucleotide conjugates, capable of detecting low concentrations of DNA in a separation-free format. This system uses QDs linked to DNA probes to capture DNA targets and successfully detect a point mutation (Zhang et al., 2005a).

Owing to their unique optical properties, QDs have also been applied for multiplex detection of analytes with single-molecule detection. Zhang et al. reported the use of a single QD-based nanosensor for multiplex detection of HIV-1 and HIV-2 at single-molecule level in a homogeneous format (Zhang and Hu, 2010). These QD-based nanosensors have several advantages such as extremely low sample consumption, high sensitivity, short analysis time and have the potential to be applied for rapid

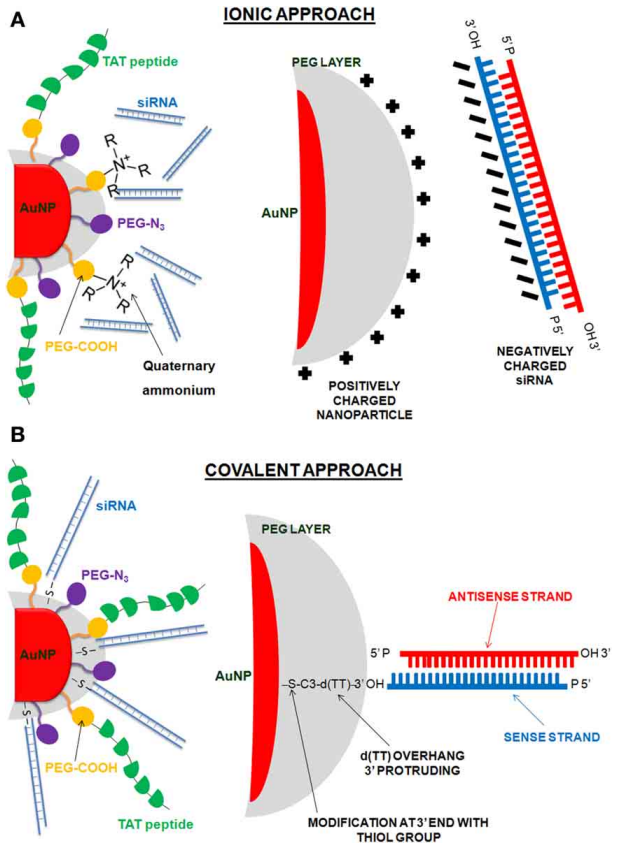

FIGURE 5 | Gold nanoparticles functionalized with multiple biomolecules: PEG, cell penetration peptide (TAT), ammonium quaternary groups, and siRNA. Two different approaches were employed to conjugate the siRNA to the AuNPs: **(A)** ionic approach, interaction of the negatively charged siRNA to the modified surface of the AuNPs through ionic interactions; **(B)** covalent approach, use of thiolated siRNA for gold thiol binding to the NPs.

point-of-care testing, gene expression studies, high-throughput screening, and clinical diagnostics.

When modified with DNA, QDs were successfully employed in the detection of respective complementary DNA strands via FRET. Sub-nanomolar detection limits have been reported (Zhou et al., 2008a; Singh and Strouse, 2010). The strategy success is directly related to the covalent coupling of the nucleic acid molecule to the QD, controlling the donor-acceptor distance, fundamental in FRET-based biosensors.

QDs can also be used in gene delivery. Jin-Ming Li et al. developed a series of QDs functionalized with β-cyclodextrin coupled to amino acids. Using the β-cyclodextrin as a vector for delivering doxorubicin (DOX) and electrostatically binding MDR1 siRNA, this strategy allowed for simultaneous chemotherapy and gene silencing. The authors observed that in HeLa cells it was possible to induce apoptosis due to the intracellular accumulation of Dox and also reduced levels of MDR1 gene expression. These multifunctional QDs are promising vehicles for the co-delivery of nucleic acids and chemotherapeutics, as well as for real-time tracking of treatment (Li et al., 2012).

QDs-siRNA conjugates have also been used for imaging and gene silencing approaches (Derfus et al., 2007; Tan et al., 2007; Yezhelyev et al., 2008; Zhao et al., 2010; Li et al., 2011, 2012). For example, Yezhelyev et al. developed multifunctional nanoparticles for siRNA delivery and imaging based on the use of QDs and proton-absorbing polymeric coatings (proton sponges). The authors demonstrated a dramatic improvement in gene silencing efficiency and simultaneous reduction in cellular toxicity, when compared with existing transfection agents. Additionally, QD-siRNA nanoparticles are also dual-modality optical and electron-microscopy probes, allowing real-time tracking and ultrastructural localization of QDs during delivery and transfection (Yezhelyev et al., 2008).

MNPs have also been frequently used as platforms for the delivery of DNA or siRNA, as they can be used to track their biodistribution by MRI. For instance, Kumar et al. synthesized multifunctional MNPs by attaching a near-infrared optical dye Cy5.5 and a peptide that targets the tumor specific antigen mucin-1 to cross-linked dextran coated SPIONs (Kumar et al., 2010). The delivery of the nanosystem to tumors in mice was imaged either *in vivo* or *ex vivo* by MRI and optical imaging.

On the other hand, the functionalization of plasmid DNA and siRNA to MNPs has been widely reported, as MNPs are used as tools for magnetofection, that is to say, the enhanced delivery of nucleic acids associated to MNPs using external magnetic fields (Scherer et al., 2002; Dobson, 2006; Plank et al., 2011). Using magnetofection the transfection efficiency can be highly improved when compared with transfections carried out with non-magnetic gene delivery systems in a variety of primary cells and cell lines (Mykhaylyk et al., 2007; Prijic and Sersa, 2011). Although magnetofection results are promising *in vitro*, and several studies have reported the systemic delivery of nucleic acids using MNPs *in vivo*, not many of them use an external magnetic field to enhance the accumulation of the MNPs in the targeted area (Plank et al., 2011). Thus far, the most promising application of magnetofection as an *in vivo* cancer therapy has been reported by Namiki et al. (2009). The authors formulated oleic acid-coated MNPs assembled with cationic lipid shells, and functionalized them with an appropriate siRNA sequence to knock down the epidermal growth factor receptor (EGFR) mRNA, as it is overexpressed in tumor blood vessel endothelium. After systemically injecting the complex to mice tumors, the authors found a 50% reduction in tumor mass when a magnetic field was applied compared to the control group without magnetic field.

Peptides

Peptides are short chains of amino acid monomers linked by amide bonds and are distinguished from proteins on the basis of size, once they only contain ∼50 amino acids or less. Peptides can be found naturally or synthetically and have the potential for the stabilization and biofunctionalization of NPs. For instance Wang et al. demonstrate that multiple functional peptide stabilized AuNPs are readily obtained in a one-step surface coating procedure and that the surface functionalities can be selectively addressed on a microarray. The authors developed a straightforward route to stable AuNPs, both with single and with dual biological functionality. The particles exhibit the specific recognition

properties of the biological cargo without any indication of non-specific binding or particle aggregation (Wang et al., 2005).

The stability conferred by peptide ligands usually depends on their length, hydrophobicity, and charge and in some cases resulted in further improved stability. Actually, Levy et al. designed a pentapeptide ligand, CALNN, which converts citrate stabilized AuNPs into extremely stable, water-soluble AuNPs with some chemical properties analogous to those of proteins. These peptide-capped AuNPs can be freeze-dried and stored as powders that can be subsequently redissolved to yield stable aqueous dispersions (Levy et al., 2004).

Biofunctional peptide sequences include "membrane translocation signals" like the HIV-TAT peptide sequence (de la Fuente and Berry, 2005; Berry et al., 2007; Conde et al., 2012a), which is capable of transporting nanoscale materials across cellular membranes and "nuclear locating signals" that could be used for further intracellular targeting (Nativo et al., 2008; Chithrani, 2010). In fact, de la Fuente et al. developed AuNPs functionalized with HIV-Tat peptide used to achieve cytoplasm and the cell nucleus. One of the major drawbacks of these cell penetrating peptides is that they are not cell specific and that they can remain entrapped in endosomes (Gump and Dowdy, 2007). To overcome these limitations, fusogenic peptides that are able to escape from endosomes or homing peptides capable of reaching specific tissues or cells have been developed (Li et al., 2004; Ruoslahti et al., 2010).

QDs labeled with these types of peptides have been extensively prepared using various strategies (Chen and Gerion, 2004; Cai and Chen, 2008; Curnis et al., 2008). For example, Akerman et al. show that ZnS-capped CdSe QDs coated with a lung-targeting peptide accumulate in the lungs of mice after intravenous injection, whereas two other peptides specifically direct QDs to blood or lymphatic vessels in tumors (Akerman et al., 2002). Curnis et al. also found that a cyclic Ciso DGRC peptide coupled to QDs could bind alphavbeta 3 integrin and co-localize with several antibodies in human renal cell carcinoma tissue sections, indicating that this peptide could efficiently recognize endothelial cells of angiogenic vessels (Curnis et al., 2008). Cai et al. also used a thiolated arginine-glycine-aspartic acid (RGD) peptide to conjugate to the QDs and applied the QDs peptide bioconjugates for tumor vasculature targeted imaging (Cai and Chen, 2008). However, if crossing the vascular wall is needed, these RGD peptides need to be improved. The Ruoshlati group reported a cyclic peptide iRGD that can combine the tumor-homing RGD sequence with a tissue penetration motif (Sugahara et al., 2009). Therefore, the homing sequence directs the peptide to the tumor vascular endothelium, while the tissue penetration motif, once activated by a protease, binds to a different receptor (neuropilin-1), which mediates extravasation and tissue penetration. As a proof of concept, iRGD peptide-linked iron oxide nanoworms could be detected by MRI throughout a tumor once injected *in vivo* to mice. Recently, the same group combined two different peptides with the magnetic nanoworms to image and treat mice with glioblastoma, one of the most difficult tumors to treat (Agemy et al., 2011). While the CGKRG peptide targets the NPs to tumor vascular cells and into their mitochondria, the other peptide acts as a pro-apoptotic drug. By co-injecting these NPs with iRGD, most of the tumors were eradicated or their development delayed in two glioblastoma mouse models.

Despite the extraordinary rapid development in strategies for nanoparticle conjugation with peptides, relatively little is known about NP behavior in the immune system, which is responsible for maintaining body integrity and preventing external invasion. Bastus et al. described the interaction between murine bone marrow macrophages and gold nanoparticle peptide conjugates. In the presence of conjugates, macrophage proliferation was stopped and pro-inflammatory cytokines were induced. Furthermore, macrophage activation by AuNPs conjugated to different peptides appeared to be rather independent of peptide length and polarity, but dependent on peptide pattern at the nanoparticle surface (Bastus et al., 2009).

Proteins/antibodies

In Bionanotechnology, specific functions of proteins such as antibody–antigen detection may also be very useful. Antibodies (Abs) or immunoglobulins are a group of proteins that have a very similar structure with four chains assembled in a Y shaped form, containing two identical domains for antigen recognition (Fab fragment), and two identical domains with effector functions (Fc fragment). The main advantage of Abs or their fragments is that the antigen-binding region is highly specific and different among Abs (Arruebo et al., 2009). Therefore, different specificity can be obtained using distinct Abs. However, attachment of Abs to the surface of NPs can impair this function if the antigen binding sites are sterically blocked upon conjugation. For this reason, the Abs orientation is extremely important to produce effective and bioactive antibody-nanoparticles.

In fact, de la Fuente et al. recently demonstrated that the combination of a good Ab orientation along with the property of a gold nanoprism to generate heat when illuminated with the correct wavelength enable visual detection of carcinoembryonic antigen (CEA) by plasmonic-driven thermal sensing with sensitivities up to the attomolar range in serum samples (Polo et al., 2013).

Immunoassays use the specificity and sensitivity of the antibody-antigen interaction in order to detect and quantify the amount of a specific analyte present in a sample. Effective conjugated antibodies can be used to constitute the desired functionality of the nanocarrier itself for immunoassays (Han et al., 2003). Actually, Putman et al. described the use of immunogold labels as cell-surface markers of human lymphocytes in atomic force microscopy. The AFM images reveal the colloidal gold particles on the cell surface, with and without silver enhancement. Individual immunogold particles are clearly resolved from the cell surface thus determining the location of antigens (Putman et al., 1993).

Ni et al. described an immunoassay readout method based on surface enhanced Raman scattering (SERS). The method exploits the SERS-derived signal from reporter molecules that are co-immobilized with biospecific species on gold colloids. This concept is demonstrated in a dual analyte sandwich assay, in which two different antibodies covalently bound to a solid substrate specifically capture two different antigens from an aqueous sample. The captured antigens in turn bind selectively to their corresponding detection antibodies. The detection antibodies are conjugated with gold colloids that are labeled with different

Raman reporter molecules, which serve as extrinsic labels for each type of antibody (Ni et al., 1999).

More recently, Conde et al. developed a highly sensitive probe for *in vivo* tumor recognition with the capacity to target specific cancer biomarkers such as EGFR on human cancer cells and xenograft tumor models. The authors used ∼90 nm AuNPs capped by a Raman reporter, encapsulated and entrapped by larger polymers and a Food and drug Administration (FDA) antibody–drug conjugate—Cetuximab (Erbitux®). These smart SERS gold nanoantennas present a high Raman signal both in cancer cells and in mice bearing xenograft tumors and the Raman detection signal is accomplished simultaneously by extensive tumor growth inhibition in mice. This approach seems to be an innovative and efficient theranostics system for both tumor detection and tumor cell inhibition at the same time (Conde et al., 2014a).

QD-Antibody conjugates have also been widely used for preparing bioconjugated QDs for *in vitro* bioassay applications (Goldman et al., 2002; Hua et al., 2006; Tan et al., 2007; East et al., 2011). In fact, Goldman et al. described the preparation and characterization of bioinorganic conjugates made with highly luminescent semiconductor CdSe-ZnS core-shell QDs and antibodies for use in fluoroimmunoassays. QD-antibody conjugates were successfully used in fluoroimmunoassays for detection of a protein toxin (staphylococcal enterotoxin B) and a small molecule (2,4,6-trinitrotoluene) (Goldman et al., 2002).

Concerning magnetic NPs, bioseparation is one of the main applications of MNP-Ab conjugates. In fact, magnetic separation of red blood cells using magnetic microspheres was reported as early as 1977 (Molday et al., 1977). MNPs are used to purify and concentrate different types of analytes in complex samples, such as hormones in biological samples, antibiotics or bacteria in food (Kuo et al., 2012; Svobodova et al., 2012; Xu et al., 2012). One of the best known systems that employ MNPs for separation and concentration is the bio-bar code technology originally described by Nam et al. (2003). In this case, Abs specific for a target protein are functionalized on the surface of MNPs, by sandwiching the target between these MNPs and an amplifier AuNPs that is loaded with a secondary Ab and oligonucleotides. When the specific target is sandwiched between the MNP and the AuNP, magnetic separation of the complexed probes allows for the concentration of the target within the sample. Afterwards, the oligonucleotides are released and detected, giving rise to a substantial amplification of the signal, and therefore lowering the detection limit to attomolar concentrations (Goluch et al., 2006). In fact, the first point-of-care nano-enabled medical diagnostic tool approved by the FDA, known as Verigene System and commercialized by Nanosphere Inc., is based on this biosensing strategy.

MNPs conjugated with Abs are also useful for the development of a new class of diagnostics nanosensors, called magnetic relaxation switches (MRS). MRS have the potential to provide sensitive and selective detection of a variety of molecular interactions with minimal or no sample preparation (Perez et al., 2002). These assays exploit the fact that when MNPs recognize and bind biological targets, they cluster, changing the spin-spin relaxation times of water protons (T2). These changes in T2 between dispersed and aggregated states of the MNPs can be monitored by nuclear magnetic resonance (NMR) (Min et al., 2012). One of the greatest advantages of these biosensors is that they employ radiofrequency radiation which penetrates biological samples regardless of their optical properties, and therefore can be used in complex samples such as blood. Using this technology, Lee et al. reported the first micro NMR biosensor, where tumor cells could be detected employing MNPs functionalized with Abs on microliter sample volumes and in multiplexed format (Lee et al., 2008). Since then, the sensitivity of these biosensors has been greatly improved using other highly magnetic MNPs (Lee et al., 2009), so that molecular profiling of cancer cells obtained by fine-needle aspirates biopsies within 60 min is possible nowadays. Using different markers, the authors reported 96% accuracy for establishing a cancer diagnosis (Haun et al., 2011).

Other proteins have also been successfully conjugated with NPs (Mattoussi et al., 2000; So et al., 2006a,b; Xia et al., 2008; Roullier et al., 2009). Mattoussi et al. first described the electrostatic interactions between negatively charged lipoic acid capped QDs and a positively charged recombinant protein (Mattoussi et al., 2000). Prasuhn et al. also developed a QD protein FRET-based biosensors used as caspase 3 proteolytic and Ca^{2+} sensors (see **Figure 6**) (Prasuhn et al., 2010).

Similar to nucleic acids, proteins are known for their specific binding interactions and can act together with a wide range of substrates and synthetic analogs. Consequently, high molecular weight peptide ligands show potential for wide biological applications and for stabilization and biofunctionalization of nanocarriers.

Enzymes

Enzymes, as highly specialized protein catalysts, are commonly used in biofunctionalization due to their potential in biotechnology and biomedicine, because of the convenience in handling, ease of separation from the reaction mixture and reuse, as well as low product cost. The immobilization in NPs often reduces diffusion limitations and/or enhances the catalytic activity of the enzymes.

An important focus of the research on AuNPs based biosensors is in enzyme electrodes. One recurrent example is glucose biosensors. Zhang et al. (2005b) described the assembly of a gold electrode modified via Au-S bond with AuNPs, where a cystamine monolayer is chemisorbed, thus exposing an array of amino groups. These are further reacted with aldehyde groups of periodate oxidized glucose oxidase via Scchiff base reaction. In this study, the NPs showed to act as conduction intermediates facilitating electron transfer, with little effect on enzyme activity. It was also shown that the sensitivity was improved as well as the affinity for glucose, hence lowering the detection limits.

In another study, MNPs were modified with N-phosphonomethyl iminodiacetic acid for immobilization of urease. Thus, the surface coating was conferred with carboxyl groups to which urease had been immobilized through carbodiimide reaction (Sahoo et al., 2011). The advantage of using MNPs is the possibility of product isolation by a permanent magnet, thus reducing costs. The authors also reported that the thermal stability of the urease was increased, showing that MNPs may be a promising material for storage and enzyme immobilization.

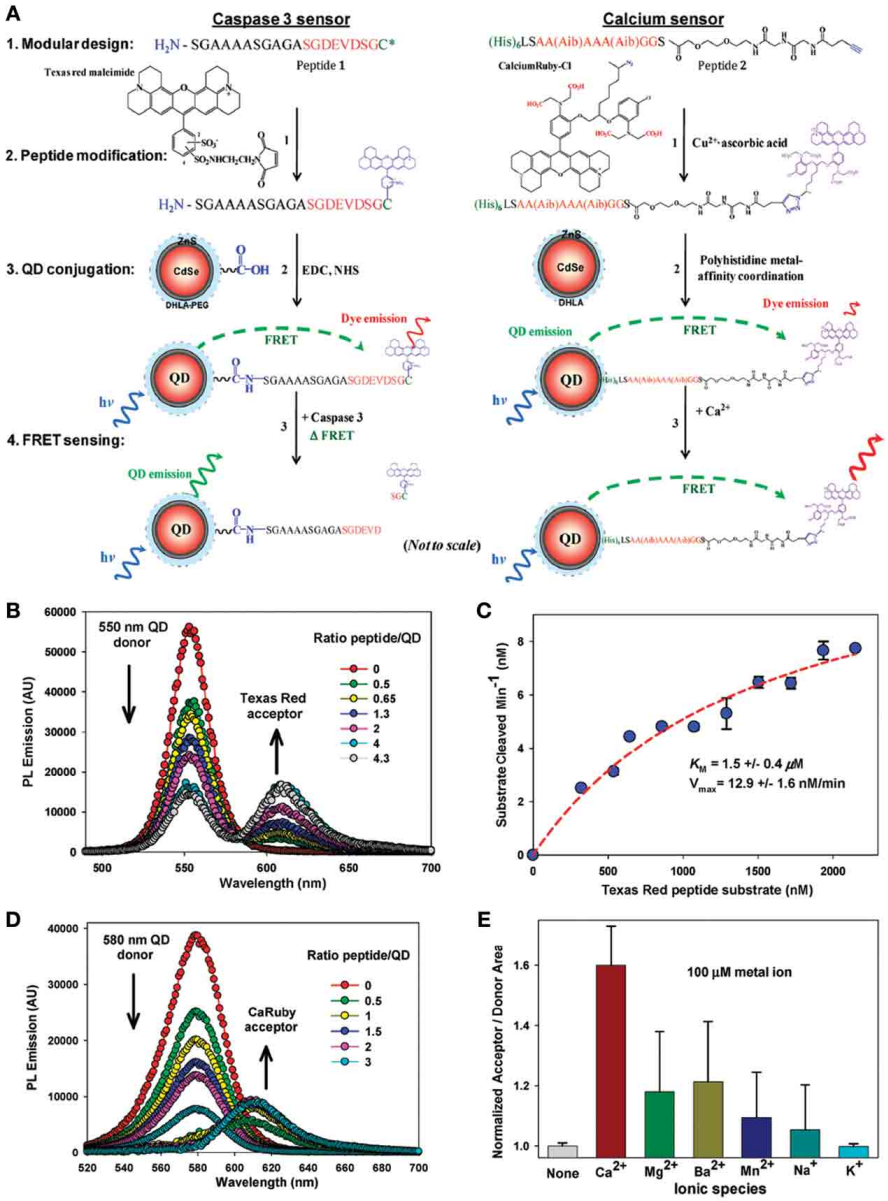

FIGURE 6 | Quantum dot protein biosensors. (A) Schematic showing the common design, chemical and sensing elements, including FRET-based biosensors: (1) peptide modularity, (2) peptide labeling, (3) attachment to QDs, and (4) FRET-based sensing for both the caspase 3 proteolytic sensor (left) and Ca^{2+} sensor (right). The 4-pendant carboxyl groups that interact with Ca^{2+} ions are shown in red on the CaRbCl structure. Within the Ca^{2+} sensor peptide sequence, Aib is the synthetic amino acid α-aminoisobutyric acid. Reactive dye structures are shown where appropriate along with the chemical linkages attaching them to the peptides. **(B)** Representative, superimposed spectra collected from 550 nm emitting QD donors surface functionalized with 85:15 DHLAPEG600-COOH/DHLA-PEG750-OMe ligands and covalently conjugated to increasing molar ratios of Texas Red-labeled substrate peptide. Samples excited at 350 nm. **(C)** Proteolytic assay data from exposing a constant concentration of 550 nm emitting QDs conjugated to 4 Texas Red substrate peptides to a constant concentration of caspase 3 enzyme. Derived Km and Vmax values are given. An $R^2 = 0.98$ was obtained for the fitting of the curve. **(D)** Representative, superimposed, and deconvoluted spectra collected from 580 nm emitting QD donors self-assembled with increasing CaRbCl-acceptor labeled peptides. Samples were excited at 350 nm. **(E)** Normalized acceptor/donor PL area ratios for 580 nm QDs self-assembled with ∼2 CaRbCl-acceptor labeled peptides exposed to selected ionic materials. The ratio from the native unexposed sensor was set to an initial value of 1 for comparison purposes (Prasuhn et al., 2010). Reproduced with permission from Prasuhn et al. (2010), Copyright 2013.

When using enzyme-based biosensors the main concern is reusability of the enzyme. Khoshnevisan et al. reported the use of MNPs to circumvent this dilemma when using cellulase. The enzyme was incubated with the MNPs and binding confirmed by FT-IR. The authors show that immobilization grants higher stability to the enzyme, thus confirming that the use of MNPs in this type of biosensors can be of great benefit (Khoshnevisan et al., 2011).

Imaging QDs *in vivo* is arduous due to the need of an external source of light, which produces strong background autofluorescence from ubiquitous endogenous chromophores. So et al. proposed the ideal QD, where it would emit light with no requirement for external excitation (So et al., 2006b). By modifying QDs with *Renilla reniformis* luciferase the authors discard the need of external excitation due to the phenomenon of bioluminescence resonance energy transfer (BRET). BRET occurs naturally and it is analogous to FRET, but the donor energy comes from a chemical reaction catalyzed by the donor enzyme. The polymer coated CdSe/ZnS core-shell QDs dotted with carboxylate groups were incubated with *R. reniformis* luciferase, where through carbodiimide reaction the amino groups of the enzyme were coupled to the carboxylates. Thus, with a simple modification the authors were able to mimic the natural BRET system with self-illuminating QDs (So et al., 2006b).

Carbohydrates

Carbohydrates are, together with nucleic acids and proteins, important molecules for life. Much is already known about the structure, interactions and function of nucleic acids and proteins, however, the role of carbohydrates in the cell is less clear (de la Fuente and Penades, 2006). A characteristic feature of the biological interactions where carbohydrates are involved is their extreme low affinity that has to be compensated by multivalent presentation of the ligands. Although individual carbohydrate interactions are relatively weak, nature utilized multivalent interactions between the cell surface ligands and their biological receptors to modulate biological events such as the ones related to cell adhesion, normal tissue growth and repair, viral/bacterial infection, signaling transduction, trapping of leucocytes, and cancer transfer. So the decoding of carbohydrate interactions opens up the possibility to employ nanoparticles in diagnostics and/or therapy (Dong, 2011). In fact, the unique physical, chemical and optical properties of the nanocarriers with carbohydrate coating comprise a series of advantages that range from ensuring water solubility, biocompatibility and stability to targeting properties (Garcia et al., 2010).

Among them, gold glyconanoparticles (glycoNPs) have drawn attention owing to their well-defined features, such as water-soluble carbohydrate-functionalized nanoclusters with a promising potential for chemical glycobiology, biomedicine, diagnostics and clinical applications. In the last 10 years, Penades and coworkers have extensively reported a pioneer integration of a glyconanotechnology strategy based on the use of nanoparticles to study and evaluate carbohydrate–carbohydrate, carbohydrate–protein interactions (**Figure 7**) (de la Fuente et al., 2001, 2006; Barrientos et al., 2003; de la Fuente and Penades, 2004, 2006), which could be used as potential tools in anti-adhesive therapy (Rojo et al., 2004), for cell–cell adhesion studies (de la Fuente et al., 2005), prevention of pathogen invasion (Reynolds et al., 2012) and for exploring blood–brain barrier permeability via neuropeptide conjugation (Frigell et al., 2014).

Smaller carbohydrates, such as lactose, glucose and mannose (Otsuka et al., 2001; Reynolds et al., 2006; Schofield et al., 2007; Martinez-Avila et al., 2009) can be thiolated for attachment to AuNPs via ligand exchange. These nanoparticles may be useful as sensitive colorimetric probes for a variety of metal ions. Mannose and lactose have also been used for the reduction of gold salts and stabilization of the nanoparticles. Schofield et al. have shown that thiolated carbohydrate derivatives can be readily assembled on silver and gold NPs. These metal glycoNPs can be used to develop aggregation based colorimetric bioassays (Schofield et al., 2006).

Magnetic glycoNPs with unique properties have also been reported, although in a more limited number (El-Boubbou and Huang, 2011; Marradi et al., 2013). Once carbohydrates are attached on the MNPs, it is crucial that they retain their biological activity. To explore this, plant lectins can be used, as their interaction with carbohydrates is highly selective. The clustering of the MNPs due to the selective recognition of the lectin can be detected using MRS assays (Moros et al., 2010).

Carbohydrates can also be used to target different cells and/or enhance the cellular uptake of NPs in a highly specific way. For instance, Moros et al. functionalized MNPs with glucose and galactose using EDC and studied their interaction with Vero cells *in vitro* (Moros et al., 2012). Although these monosaccharides share the same chemical formula, except for the spatial conformation of the hydroxyl group in C-4, the cell entrance pattern was completely different. While MNPs-glucose entered all throughout the cell, MNPs-galactose remained predominantly in the cell periphery. By preparing a library of MNPs functionalized with different monosaccharides, El-Boubbou et al. were also able to detect, differentiate cancer cells and quantitatively profile their carbohydrate binding abilities by MRI (El-Boubbou et al., 2010).

Carbohydrates have been also conjugated to QDs (Chen et al., 2003; Osaki et al., 2004; Kikkeri et al., 2009; Cai et al., 2012; Yang et al., 2012a). For example, Kikkeri et al. synthesized PEGylated QDs capped with D-mannose, D-galactose, and D-galactosamine to study specific carbohydrate-protein interactions *in vitro* and *in vivo*. These QD-carbohydrates were produced through covalent coupling by 4-maleimidopropanoic acid NHS ester and used for *in vitro* imaging and *in vivo* liver targeting (Kikkeri et al., 2009). Shinchi et al. also developed glycol-QDs by preparing stable sugar-chain-immobilized fluorescent nanoparticles (CdTe/CdS core/shell QDs functionalized with sugar-chain-ligand conjugates, β-galactose- and α-glucose) and their application to the analysis of sugar-chain-protein interactions and cellular imaging (Shinchi et al., 2012).

BIOMOLECULE COUPLING STRATEGIES

Functionalization of NPs with biomolecules has to face several hurdles and surface modifications can have significant impacts on their physical-chemical properties and therapeutic efficacy, once they might alter surface charge, size, hydrophobicity, and targeting skills. One of the biggest challenges is that NPs need to remain stable in solution while the conjugation takes place. However, many NPs may precipitate while being activated, as their stability depends on a delicate balance between attractive and repulsive forces, which can be modified when using different chemicals for their biofunctionalization. Moreover, due to the huge amount of different NPs and biomolecules reported so far, there are no standardized protocols for NP functionalization. Therefore, the choice of a coupling strategy depends on the

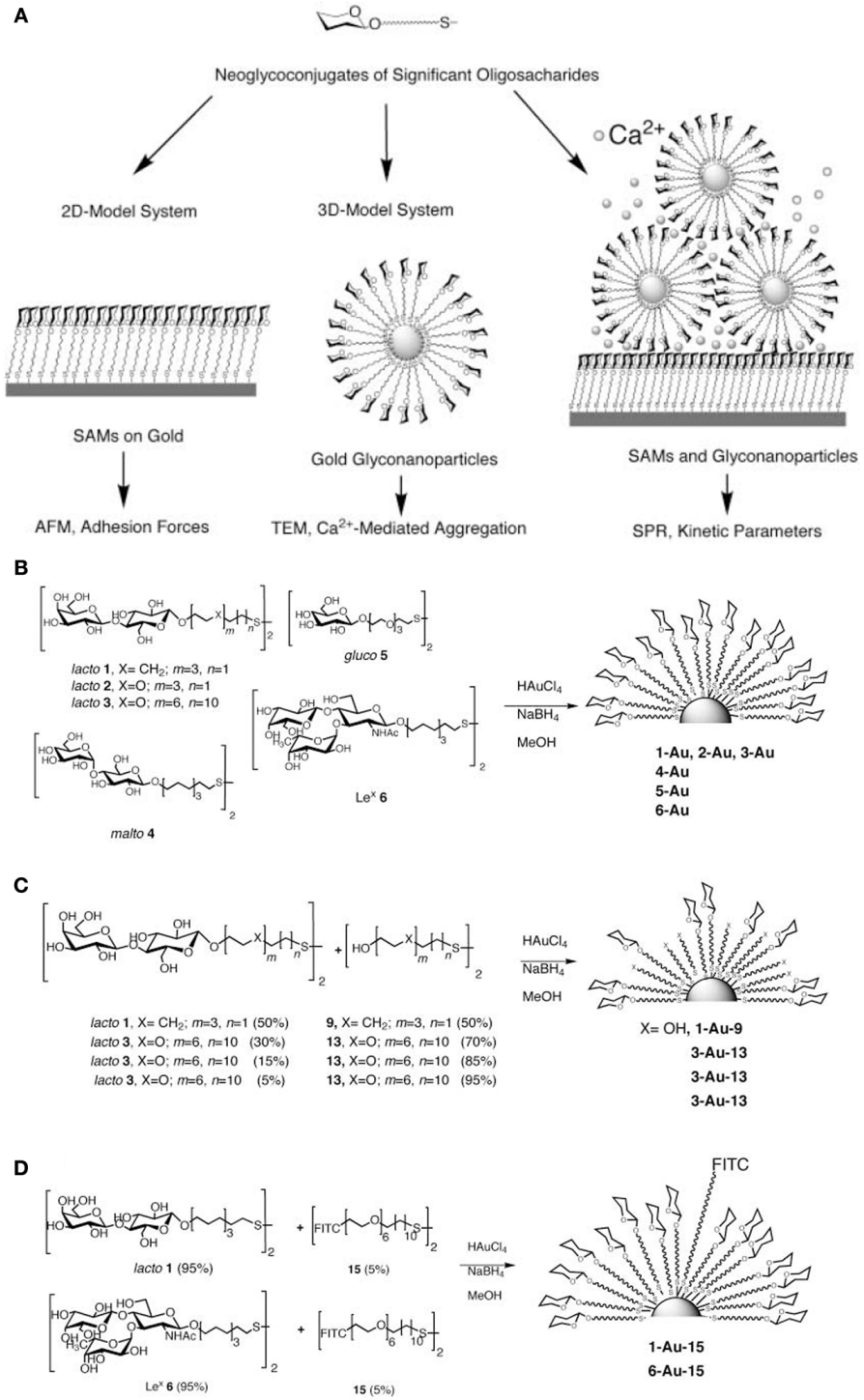

FIGURE 7 | Gold glyconanoparticles. (A) Strategy for studying carbohydrate ± carbohydrate interactions based on 2D and 3D models that mimic carbohydrate presentation at the cell surface. Preparation of **(B)** glyconanoparticles; **(C)** hybrid glyconanoparticles; **(D)** fluorescence glyconanoparticles (Barrientos et al., 2003). Reproduced with permission from Barrientos et al. (2003), Copyright 2013.

stability of the NP, the functional groups, the bioconjugation conditions (pH, temperature, ionic strength, solvent choice, structure of the surfactant) and the biomolecule to attach, among others. Finally, depending on the conjugated biomolecule, it is important to control the orientation, so that the biomolecule remains active once conjugated to the NP.

Biofunctionalization can be achieved using several techniques, such as physical adsorption and electrostatic binding, specific

affinity recognition, and covalent coupling, each of which has its own advantages and disadvantages. In this section of the review, the several coupling strategies for biofunctionalization of gold, MNPs and QDs will be examined.

Covalent strategies

Covalent coupling provides stable and strong binding of the biomolecules to the NPs. Most proteins have amine groups in their surface, so they can be directly conjugated to NPs containing reactive groups such as aldehydes, epoxides or anhydrides (Fuentes et al., 2005; Lu et al., 2008).

On the other hand, coupling of NPs that exhibit amine groups with molecules containing aldehydes or epoxides can also be used. However, some biomolecules such as antibodies, oligonucleotides, carbohydrates or peptides do not include these functional groups, and should be modified prior to the conjugation (Nobs et al., 2004). For instance, carbohydrates present in some antibodies can be oxidized using periodate to generate aldehyde groups that can react with the amino groups present on the NPs surface (Fuentes et al., 2005). Nevertheless, chemical modification may compromise biomolecules' activity, so one of the most frequent ways to conjugate molecules to the NPs is using linker molecules.

EDC coupling reaction. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) is a zero-length crosslinking agent used to couple carboxyl or phosphate groups to primary amines, which may react with a carboxyl group of a biomolecule, forming an amine-reactive O-acylisourea intermediate. Addition of sulfo-NHS stabilizes the amine-reactive intermediate by converting it to an amine-reactive sulfo-NHS ester. The O-acylisourea intermediate may also react with an amine on a second biomolecule, producing a conjugate of the two biomolecules joined by a stable amide bond. This crosslinker has been used in diverse applications, such as conjugation of carboxyl to amine groups in peptides and proteins, forming amide bonds in peptide synthesis, attaching haptens to carrier proteins and form immunogens, labeling nucleic acids through 5′ phosphate groups and creating amine-reactive NHS-esters of biomolecules (Grabarek and Gergely, 1990).

One of the main advantages of EDC coupling is water solubility, which allows direct bioconjugation without prior organic solvent dissolution. On top of that, the excess of reagents and by-products can be easily removed by dialysis or gel-filtration (Sheehan et al., 1965). However, the coupling reaction has to be carried out fast, as the reactive ester that is formed can be rapidly hydrolyzed in aqueous solutions. To increase the stability of this active ester, N-hydroxysuccinimide (NHS) or N-hydroxysulfoxuccinimide (sulfo-NHS) can be used (Jang and Keng, 2008). Key parameters that should be controlled when using EDC are pH (as hydrolysis is largely dependent on pH), the amount of EDC so that NPs do not aggregate due to loss of electrostatic repulsive forces between NPs, and the ratio EDC/NHS (Nakajima and Ikada, 1995; Sam et al., 2009; Shen et al., 2009).

Using this protocol almost all kinds of molecules (i.e., enzymes, antibodies, peptides, DNA, fluorophores, etc.) may be attached to the nanoparticle surface without prior modification

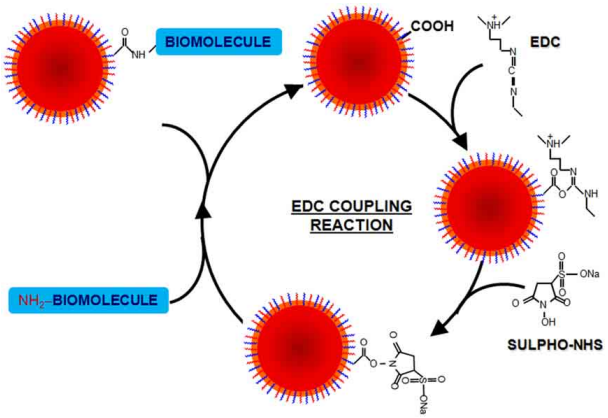

FIGURE 8 | EDC coupling reaction. The 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) is a zero-length crosslinking agent used to couple carboxyl groups to primary amines. In the presence of N-hydroxysulfosuccinimide (Sulfo-NHS), EDC can be used to convert carboxyl groups to amine-reactive Sulfo-NHS esters. The addition of Sulfo-NHS stabilizes the amine-reactive intermediate by converting it to an amine-reactive Sulfo-NHS ester, thus increasing the efficiency of EDC-mediated coupling reactions. Excess reagent and crosslinking by products are easily removed by washing with water. Once EDC is water soluble, the crosslinking can be done under physiologic conditions without adding organic solvent.

(see **Figure 8**) (Pandey et al., 2007; Susumu et al., 2007; Rostro-Kohanloo et al., 2009; Conde et al., 2012a; Lavilla et al., 2012). For instance, using the EDC chemistry, Weissleder and coworkers created a library of MNPs decorated with different synthetic small molecules for the development of magnetofluorescent reporters (Weissleder et al., 2005). Using these fluorescent MNPs it was possible to screen against different cell types or among different physiological states of a cell line. On the other hand, Sanz et al. have reported the effect of biofunctional spacers, such as thiolated PEG chains on the loading of RNA molecules and a positive peptide functionalized by EDC coupling reactions on the surface of AuNPs (Sanz et al., 2012). Lin and coworkers also used EDC to attach CH_3O-PEG-NH_2 to different types of carboxylated NPs (MNPs, QDs) demonstrating that adjusting the ratio EDC/NP it was possible to prepare NPs with 0, 1, or 2 attached PEG molecules (Lin et al., 2008). Similarly, Parak and co-workers also used EDC to attach NH_2–PEG–NH_2 molecules, varying the molecular weight of the polymer on the surface of AuNPs (Sperling et al., 2006; Pellegrino et al., 2007). The covalent attaching of biofunctional short PEG molecules to the polymer shell produces very stable particles in electrolytic solution. This approach results in stable water-soluble AuNPs (Sanz et al., 2012) and QDs (Ballou et al., 2004) with functional groups, e.g. $-$COOH or $-NH_2$ on the free ends of PEG molecules. By controlling the EDC ratio, aggregation was prevented. Dhar et al. have exploited the rapid intracellular uptake of AuNPs to deliver and activate cisplatin and achieve efficient cytosolic delivery of platinum(IV) prodrug to lung cancer cells. The AuNPs used in this study were functionalized with thiolated oligonucleotides containing a terminal dodecyl amine for conjugation

with a platinum(IV) compound capable of being tethered to an amine functionalized DNA-AuNP surface via amide linkages (Dhar et al., 2009).

Similarly, conjugation of biomolecules to QDs through coupling reactions with reactive functional groups presented on QDs surface is another common strategy to prepare QD bioconjugates. Actually, carboxylic acid groups can also be added to QDs' surface and subsequently conjugated to biomolecules with primary amine groups through EDC coupling reactions (Cai et al., 2006; Hua et al., 2006; Choi et al., 2009; Wu et al., 2009a; East et al., 2011).

One disadvantage of this type of chemistry is that the presence of both carboxylates and amines on one of the biomolecules to be conjugated with EDC can result in self-polymerization and consequently, loss of effectiveness. For instance, peptides usually contain both types of groups, so if EDC is added in the presence of them, peptides can polymerize. However, Bartczak et al. have used this strategy to coupling of peptides to AuNPs in a one-pot way. The authors have shown that the concentration, reaction time, and chemical environment are all critical to achieving the formation of robust, peptide-coated colloidal nanoparticles without aggregation (Bartczak and Kanaras, 2011). Another way to avoid polymerization of the biomolecule is to eliminate the excess of EDC before adding the biomolecule to the NPs solution by magnetic separation in the case of MNPs or gel-filtration for other NPs.

Despite the simplicity of this technique, that does not require prior chemical modification of the biomolecule, it does not guarantee an oriented immobilization in the case of biomolecules with greater structural complexity, such as antibodies. Due to the poor stability of the reactive ester, neutral pH is traditionally used to link covalently antibodies to carboxylated-NPs. At this pH, immobilization mainly occurs through direct covalent binding of the most reactive amine groups of the antibody. Unfortunately, these are the terminal amine moieties of the four Ab polypeptide chains (pKa around 7–8), which are all located in the antigen-binding domain (Puertas et al., 2011). Recently, Puertas et al. described a smart approach that takes advantage of the existing kinetic differences among ionic adsorption processes and covalent reactions in order to assure the oriented covalent attachment of the Ab using EDC chemistry. Briefly, it requires the selection of the best incubations conditions (pH, ionic strength) to promote a fast ionic adsorption of the Ab due to the negative charges of the carboxylic groups of the NP. This ionic adsorption makes possible the orientation of the Ab on the NP surface before irreversible covalent bond formation (**Figure 9**) (Puertas et al., 2011). Initially, the authors optimized this two-step strategy for magnetic NPs but they have recently extended

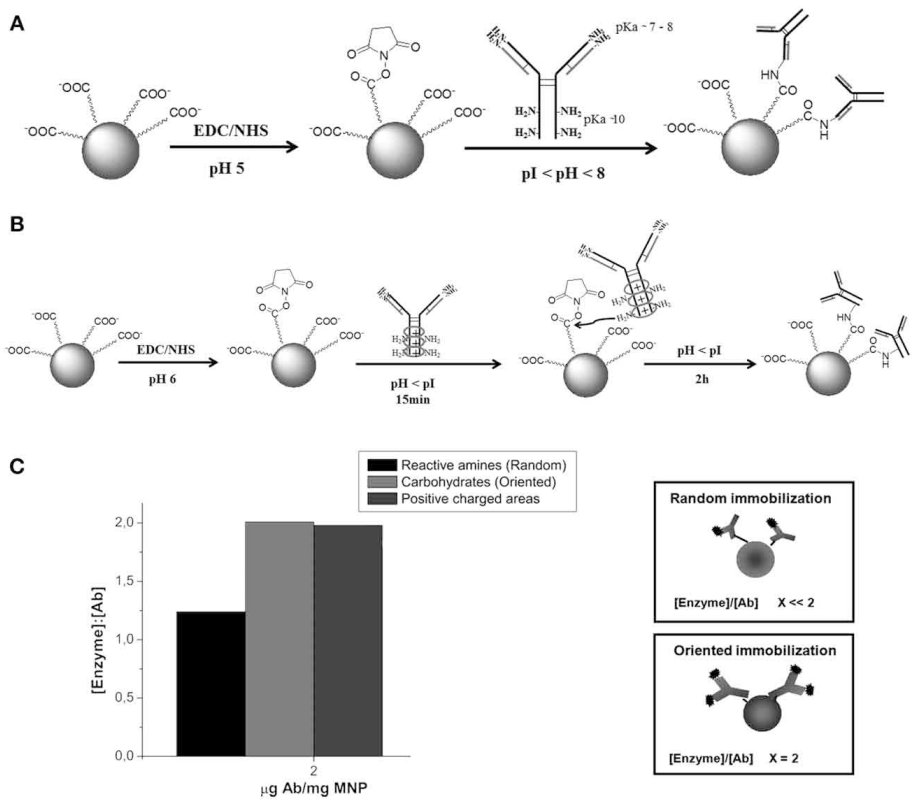

FIGURE 9 | Highly active magnetic nanoparticle-antibody conjugates. **(A)** Two-step immobilization mechanism proposed when using Ab that bind to the MNPs through the most reactive amines—random immobilization. **(B)** Covalent attachment via the polysaccharide moieties of the antibody to the MNPs—oriented immobilization. **(C)** Capacity to capture HRP of the anti-HRP anchored to MNPs by different orientations. The protein content of all anti-HRP-functionalized MNPs was similar (2 µg Ab per mg MNP) (Puertas et al., 2011). Reproduced with permission from Puertas et al. (2011), Copyright 2013.

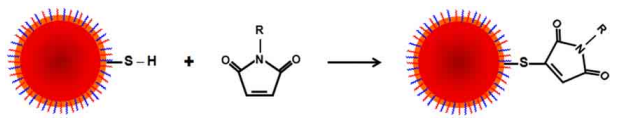

FIGURE 10 | Maleimide coupling reaction. Maleimide reacts with free sulfhydryl group(s), forming stable thioether linkages, at physiological pH. It is useful for bioconjugation of proteins with −SH groups and the coupling of two thiols to form a disulfide linkage.

application for functionalization of other nanostructured materials, such as gold nanoprisms and carbon nanotubes (Polo et al., 2013).

Maleimide coupling. Maleimide can be used to conjugate primary amines to thiols (Brinkley, 1992) (see **Figure 10**). The use of maleimide for modification of sulfhydryl groups has been extensively described in the literature (Means and Feeney, 1990). Reaction with sulfhydryl groups generates a stable 3-thiosuccinimidyl ether linkage and occurs normally at pH 6.5–7.5. One of the main limitations is that the maleimide ring may hydrolyze in aqueous buffer to a non-reactive cis-maleamic acid derivative over long reaction times or at pH > 8.0. Nevertheless, this type of conjugation shows a lot of potential for a great number of biomolecules that bear reactive thiol or amino groups. This may eventually lead to non-specific bonds and crosslinking between functionalized nanoparticles since a single biomolecule may have several thiol groups (Means and Feeney, 1990; Brinkley, 1992).

Maleimide coupling has been used to conjugate several biomolecules to AuNPs, such as peptides (Oh et al., 2010a; Ravi et al., 2012), chemotherapeutic agents (Hwu et al., 2009), dyes (Zhu et al., 2012a), and DNA (Lee, 2011). In fact, Ba et al. presented a versatile and controlled route to immobilize AuNPs on the surface of living cells, while preserving the sensing and optothermal capabilities of the original colloid, by chemically anchoring the nanoparticles to phospholipids in liposomes via maleimide-thiol reactivity (Ba et al., 2010).

Maleimide coupling was also used to couple DNA (Dubertret et al., 2002), PNAs (Srinivasan et al., 2006), proteins (Wolcott et al., 2006; Bonasio et al., 2007; Zhou et al., 2007), and antibodies into QDs (Diagaradjane et al., 2008). To address biocompatibility issues of QDs, Dubertret et al. encapsulated individual nanocrystals in phospholipid block-copolymer micelles conjugated to DNA and demonstrated their function as fluorescent probes (Dubertret et al., 2002). Bonasio et al. also reported the specific and covalent labeling of QDs with a membrane protein and organic fluorophores (Bonasio et al., 2007).

Similarly, MNPs can also be functionalized using the maleimide coupling reaction with PEG (Kuhn et al., 2006), DNA (Nam et al., 2004) or even drugs, such as chlorotoxin (Kievit et al., 2010). Concerning antibodies, this chemistry could be also used with thiol or amino groups on the nanoparticle surface (Lee et al., 2007; Haun et al., 2010). Regarding a thiolated NP, antibodies would bind through their most reactive amine groups and, as previously explained, this could lead to a random orientation with partial loss of the Ab's biological activity. Instead, maleimide chemistry used with aminated NPs ensures an oriented binding through thiol groups of the Ab. However, as in Abs sulfhydryls are oxidized as disulfides. So it is necessary to selectively reduce the disulfides at the hinge region by a reducing agent (i.e., 2-mercapthoethylamine, mercaptoethanol, dithiotreitol, thiopropyl-agarose). This chemical modification can also be combined with fragmentation of the IgG by the use of proteolytic enzymes (i.e., pepsine, ficin) in order to conjugate small Ab fragments such as F(ab′)2 and Fab′.

Click-chemistry reaction. The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) click reaction has been recognized as a facile and versatile chemistry for bioconjugation. Azides and alkynes are highly energetic functional groups with particularly narrow distributions of reactivity. Thanks to their weak acid-base properties, they are nearly inert toward biological molecules and toward the reaction conditions found inside living cells. The azide groups are easy to introduce into organic compounds by both nucleophilic and electrophilic processes. One of the most common bioconjugation of azides is the copper catalyzed azide-alkyne cycloaddition (CuAAC) (Wang et al., 2003) (see **Figure 11**). This reaction features an enormous rate acceleration of 10^7–10^8 compared to the uncatalyzed 1,3-dipolar cycloaddition (Himo et al., 2005). This reaction has also been termed the "cream of the crop" of click reactions and is surely responsible for the tremendous popularity of the "click" concept and many simply associate "click chemistry" to mean triazole formation between an azide and alkyne. The reaction occurs at room temperature, showing a high degree of solvent and pH insensitivity, and high chemoselectivity (the azide and alkyne are inert to react with numerous functional groups under the typically mild reaction conditions). In fact, the reaction succeeds over a broad temperature range, is insensitive to aqueous conditions and occurs in a pH range between 4 and 12 (Hein and Fokin, 2010; Le et al., 2010). The copper catalyzed azide-alkyne cycloaddition occurs between an organic azide and a terminal acetylene. The cyclic product is a triazole. The copper catalyst allows the reaction to proceed at room temperature and confers regioselectivity (a reaction in which one direction of bond making or breaking occurs preferentially over all other possible directions), with the 1,4 regioisomer being the only product. The reaction start by the incubation with a mixture of copper(II) (e.g. copper(II) sulfate) and a reducing agent (e.g. sodium ascorbate) to produce Cu(I) *in situ* (Meldal and Tornoe, 2008; Hong et al., 2009).

Click chemistry sometimes refers to a group of reactions that are fast, simple to use, easy to purify, versatile, regiospecific, and give high product yields. However, the click reaction has a number of limitations. First, like with any cycloaddition, if the azide group is too electron deficient, then it will not undergo the reaction. In other words, the ground state configuration of the azide is far too low to interact with the terminal alkyne (Hein et al., 2008). Secondly, a more common problem is alkyne homocoupling. This phenomenon occurs when an alkyne reacts with a second alkyne instead of the azide. This process can be minimized by using a sterically bulky base that stabilizes the reactive intermediates of the homocoupling reactions. The Cu(I) saturation is rare but can also be a problem, once the alkynes may

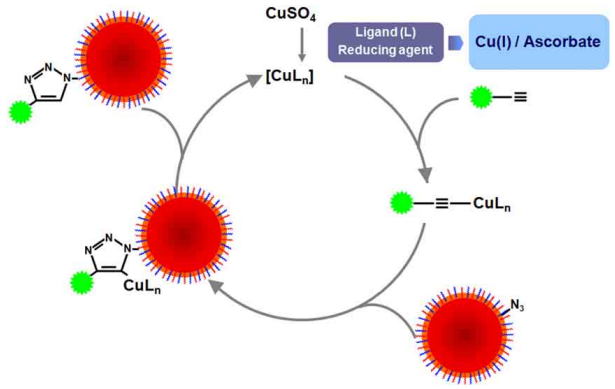

FIGURE 11 | Click chemistry reaction. The copper-catalyzed cycloaddition of azides and alkynes (CuAAC) developed for click chemistry joins an organic azide (N_3) and alkyne together producing a mixture of 1,4- and 1,5-triazoles.

chelate the Cu(I)-acetylide complex intermediate that contact with the azide group (Hein et al., 2008). Besides these limitations, one of the most obvious disadvantages is the requirement of a copper catalyst. In fact, an excessive intake of copper can lead to drastic consequences for the human body (e.g. hepatitis, neurological disorders, kidney diseases and Alzheimer's disease) (Wang and Guo, 2006; Hein et al., 2008).

Generally, the click-chemistry reaction has been used to couple AuNPs to proteins (Zhu et al., 2012b), enzymes (Brennan et al., 2006; Kim et al., 2010), fluorophores (Voliani et al., 2011), polymers (Boisselier et al., 2008; Zhang et al., 2009), and other small molecules (Fleming et al., 2006). For example, following click-chemistry reaction Fleming et al. were able to conjugate to AuNPs several different alkyne derivatives, such as ferrocene, aniline and PEG (Fleming et al., 2006).

Alkyne-functionalized AuNPs have been also extensively used to detect metal ions in aqueous solutions, such as Cu^{2+}, using click chemistry. This method allows visualization by naked eye of the presence of Cu^{2+} ions by the aggregation of AuNPs as a result of the Cu(I)-catalyzed conjugation between the two functional groups (Zhou et al., 2008b; Xu et al., 2010; Lin et al., 2012).

Another common type of nanoparticles used for click-chemistry bioconjugation is QDs. QDs need to be coated to other chemical species if they are to be used as biomarkers, therapeutic agents or sensors. In fact, water soluble and water QDs have been successfully coated with polymers via click-chemistry reactions (Beaune et al., 2011; Janczewski et al., 2011; Lai and Guan, 2011; Petryayeva and Krull, 2012; Zhang et al., 2012a). Jańczewski et al. reported the use of click-QDs by producing water solubilization of hydrophobic CdSe/ZnS QDs using amphiphilic polymeric coatings. The authors described the preparation of acetylene- and azide-functionalized QDs for "click" chemistry. The method is universal and applicable to any type of nanoparticle stabilized with hydrophobic ligands able to interact (in water) with the alkyl chains present in the coating (Janczewski et al., 2011).

Interestingly, Hao et al. reported a method for labeling viruses via copper-free click chemistry to QDs. The authors linked virions modified with azide to QDs capable of realizing single-virion tracking, laying the foundation for long-term dynamic visualization of virus infection process (Hao et al., 2012).

Although click chemistry does not appear to be a major type of chemistry for MNPs, some interesting examples can be found in the literature. For instance, Santra and coworkers reported the creation of novel polymeric-metallic nanocomposites when assembling alkylated IONPs with azide polymer fluorescent NPs, obtaining a fluorescence material with enhanced magnetic properties for MRI (Santra et al., 2009). The first example of click MNPs for *in vivo* applications was reported by Bhatia and coworkers (von Maltzahn et al., 2008). Fluorescent MNPs functionalized with a tumor-targeting peptide (Lyp-1) *via* click chemistry were able to stably navigate the systemic circulation, extravasate into tumors and penetrate into the interstitial space to specifically bind to receptors on tumor cells. Weisledder group also reported the introduction of ^{18}F onto azide-modified MNPs using click-chemistry for *in vivo* PET imaging (Devaraj et al., 2009).

Non-covalent strategies: physical interactions

Physical interactions include electrostatic, hydrophobic and affinity interactions. These interactions have several advantages, such as the ease of functionalization, speed of binding and that neither the biomolecules nor the NPs must be modified in case of electrostatic and hydrophobic interactions. However, conjugation is less stable and reproducible when compared to covalent methods. Moreover, it is difficult to control the amount and orientation of bound molecules.

Ionic coupling. Ionic adsorption provides a simple and straightforward method to functionalize NPs with biomolecules. In fact, biological and polymeric species with an opposite charge can be coupled to NPs (Conde et al., 2012a) or between different opposite charged NPs (Liu et al., 2012). Ionic binding rate mainly depends on the amount of charges present on the NPs and the biomolecules, as the binding is made by multiple point (multipunctual). Therefore, when binding complex molecules such as antibodies or proteins, the isoelectric point should be considered, as their net charge would depend on it.

Ionic coupling has been traditionally used to adsorb proteins to NPs (Brewer et al., 2005; Hong et al., 2006; Reed and Metallo, 2010; Guo et al., 2011; Brancolini et al., 2012; Strozyk et al., 2012), as some proteins such as serum albumin can stabilize NPs by preventing aggregation (Brewer et al., 2005). Moreover, proteins can be adsorbed to NPs to increase cellular uptake or specificity toward tumor cells (Chang et al., 2012). Negatively charged hyaluronic acid (HA) was also used to self-assemble onto the positively charged QDs through ionic interactions. For this, Bhang et al. developed a simple and novel electrostatic coupling method, which provides a HA-QD conjugate with cancer targeting efficiency to use in diagnostic and imaging applications. These conjugates were also effective for fluorescence staining of lymphatic vessels *in vitro* and *in vivo* (Bhang et al., 2009).

Despite the ease of this conjugation method, the native structure of the adsorbed proteins may be affected (Lacerda et al., 2010), which could ultimately result in loss of biological activity or even cellular toxicity (Vertegel et al., 2004; Deng et al., 2011).

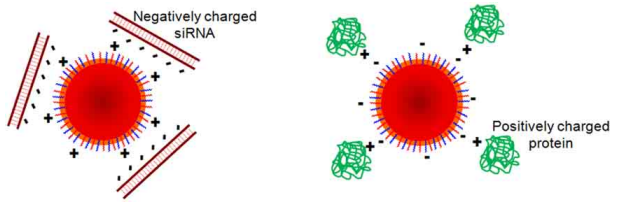

FIGURE 12 | Ionic coupling. Coupling of negatively charged siRNA (left) and of positively charged proteins (right) to negatively or positively charged AuNPs.

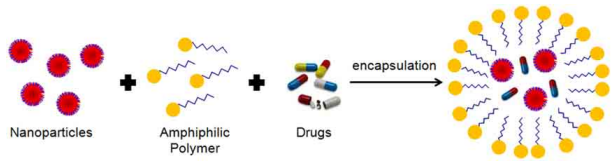

FIGURE 13 | Hydrophobic coupling. Use of amphiphilic polymers as nanoparticle and drug delivery moieties.

Other examples of biological applications are the coupling of negatively charged DNA (Thomas and Klibanov, 2003; Ghosh et al., 2008; Conde et al., 2012b) or siRNA (Elbakry et al., 2009; Guo et al., 2010; Conde et al., 2012a; Zhao et al., 2012a) to positively charged NPs (**Figure 12**) (Huschka et al., 2012). In fact, Conde et al. reported functionalization of siRNA by ionic coupling to a positively charged layer formed by quaternary ammonium groups (R_4N^+). Ionic interactions between the negatively charged siRNA backbone (via phosphate groups) and quaternary ammonium positively charged groups ensured binding of siRNA onto the AuNPs' surface for the whole pH range. Using a hierarchical approach including three biological systems of increasing complexity, *in vitro* cultured human cells, *in vivo* freshwater polyp (*Hydra vulgaris*) and *in vivo* healthy mice model, these authors identified the most adequate nanoparticles to efficiently transport siRNAs. The results evidenced the importance of a correct design in the functionalization of nanoparticles for biological applications, in particular for complex animal systems, such as mice. The ionic linkage of siRNA on the AuNPs showed efficiency in cells and in *Hydra*. However, only a covalent bond ensured an active and efficient RNAi mechanism in mice (Conde et al., 2012a). Similarly, Li et al. reported the development of QDs-DNA complexes that are disrupted and DNA released by glutathione (GSH) at intracellular concentrations (Li et al., 2008).

As extensively reviewed by others (Montenegro et al., 2013), conjugation of Abs to NPs may be made with covalent immobilization techniques such as those mentioned before, but also with non-covalent strategies. The most common technique is the electrostatic adsorption of Abs by charge interaction to opposite charged NPs. This ionic adsorption is directly related to the Abs isoelectric point, pH at which they are neutral. Since it depends mainly on the number of charged groups, the Abs immobilized region will be where the greatest number of charges are present (Jung et al., 2008). This method, although of easy implementation, shows several disadvantages. The main concern is the weak pH dependent interaction between the Ab and NP. Any changes to the pH and/or ionic strength may incur in desorption of the Abs molecules. Additionally, the heterogeneous charge distribution and the unexploited charged groups of the Abs can promote non-specific adsorption to matrix proteins, for example competitive displacement caused by serum proteins (van der Voort et al., 2004; Murcia and Naumann, 2005).

Hydrophobic coupling. Hydrophobic interactions have been widely exploited to attach lipophilic drugs to NPs, from where the drug might be released once inside the cells (Wahajuddin and Arora, 2012). For instance, docetaxel has been easily adsorbed to the oleic acid layer that surrounded hydrophobic MNPs prior to their encapsulation in a polymeric vesicle, providing a controlled drug release profile for a month (Ling et al., 2011). Encapsulation of drugs can also be done adsorbing them to hydrophobic polymers or cyclodextrins for instance (Yallapu et al., 2011) (see **Figure 13**).

Similarly, hydrophobic molecules such as fluorophores can be coupled to NPs. Foy and coworkers coated hydrophobic NPs with amphiphilic polymers, to which they adsorbed five different dyes to determine MNP biodistribution using a mouse xenograft breast tumor model (Foy et al., 2010). Moreover, Kim et al. developed AuNPs functionalized with water-soluble zwitterionic ligands from kinetically stable complexes with hydrophobic drugs and dyes, which are efficiently released into cells (Kim et al., 2009). This coupling method minimizes changes to the surface and allows the creation of NP with dual properties, such as optical and therapeutic, in an easy way.

Proteins and Abs can also be adsorbed to NPs *via* hydrophobic interactions. However, they often suffer from denaturation, leading to poor reproducibility. Hydrophobic interactions with the hydrophobic surfaces of proteins or Abs force a change in the native structure because of exposure of its inner region, which could ultimately results in loss of activity (Zuo et al., 2010; Shemetov et al., 2012). Moreover, regarding ionic binding, controlling the orientation or the amount of bound molecules is difficult to achieve.

Biotin-avidin system. For NPs conjugation, factors such as solubility, charge and all the aforementioned functional groups confer the biotin a relevant importance (Aslan et al., 2004). Biotinylation of NPs and biological molecules nowadays is fairly common as biotin can be synthesized to have a distal amine, thiol, carboxyl and other functional groups, simplifying the conjugation (Hermanson, 2008). However, to avoid a random immobilization of structural complex biomolecules such as proteins, it is compulsory to achieve a site specific biotinilation. Concerning antibodies, for example, biotinilation must be carried out within its Fc region via the carbohydrates moieties or via thiols obtained after reduction of the disulfides located in the hinge region (Cho et al., 2007).

Genetic engineering has also improved biotinylation by recombinantly introducing biotin labeling sites into fusion proteins (Cronan, 1990; Cull and Schatz, 2000). Nowadays several companies offer biomolecules and NPs modified with biotin or avidin species. The high-affinity of the avidin-biotin interaction

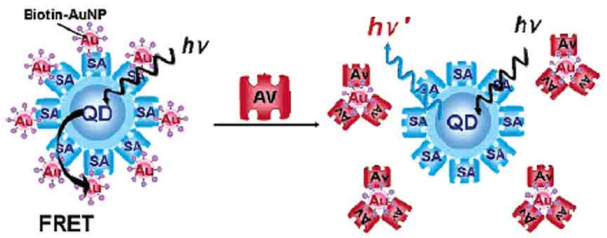

FIGURE 14 | Biotin-avidin system. Schematic of inhibition assay method based on the photoluminescence quenching of SA-QDs by Biotin-AuNPs. SA denotes the streptavidin immobilized on the surface of QDs, and Av is the externally added avidin (Feng et al., 2013). Reproduced with permission from Feng et al. (2013), Copyright 2013.

(K_d around 10^{-14}) has made it perfect for the development of NPs-based biosensors. The main method of bioconjugation using avidin-biotin chemistry comprises the functionalization of NPs with avidin, for later incubation with a biotinylated molecule. Since avidin and its variants are zwitterionic molecules, they can be subject to electrostatic adsorption to negatively charged nanoparticles (see section Non-Covalent Strategies: Physical Interactions). The vast number of publications that apply biotin-avidin interaction for bioconjugation shows the importance of this strategy. Recently, Feng et al. showed that DNA detection could be improved using streptavidin coated AuNPs (Feng et al., 2013). Another interesting strategy was reported by Oh et al. where by modulating the FRET efficiency between QDs and AuNPs they were able to detect molecules which inhibited the interaction between streptavidin and biotin (**Figure 14**) (Oh et al., 2005). By capping the AuNPs with polyamidoamine dendrimers, the biotinylation was possible using sulfo-NHS-biotin.

A similar strategy was employed in the biotinylation of MNPs. To achieve a MNPs-PEG-biotin conjugate the NPs were incubated with a phospholipid-PEG-biotin construct. By coupling $DC_{14:0}$ PE (dimyristoylphosphatidylethanolamine) to further activate α-biotinylamido-ω-N-hydroxy-succinimidcarbonyl-PEG, the authors could produce MNPs covered with PEG-biotin. The functionalization was confirmed when binding streptavidin alkaline phosphatase the complexes became highly aggregated (Hodenius et al., 2012).

The strong association between avidin and biotin has made this system a reference for the development and troubleshooting of NPs-based biosensors. It is also a crutch for conjugation of other biomolecules onto the surface of NPs. However, it is important to note that avidin is a glycoprotein with a high isoelectric point (∼10). This could cause the unspecific binding of other compounds present in complex biological samples. To overcome this problem, it is preferred the use of streptavidin. As it is purified from a bacteria (*Streptomyces avidinii*) is not a glycoprotein and has much lower isoelectric point (around 5–6). Besides, the tetrameric nature of each (strept) avidin molecule becomes a problem when control of the Ab stoichiometry is needed. To overtake this problem, it is possible to use recombinant monomerics forms of these proteins but taking into account that the affinity for biotin would be much lower (around 10^{-7} M) (Wu et al., 2009b).

CONCLUSIONS AND FUTURE PERSPECTIVES

In the near future, it is expected that the design of nanosystems will revolutionize the medical healthcare field by their application in the development of ultrasensitive and multiplexed diagnostic systems, targeted and remotely controlled drug delivery systems for treatment of diseases, *in vivo* imaging, tissue/organ regeneration and gene therapy solutions. The last three decades have been an exciting period in the synthesis of inorganic nanoparticles with interesting intrinsic properties for their use in such applications. Indeed, many of these synthetic processes have not only demonstrated proof-of-concept feasibility but progressed to full-scale commercial production. However, optimization of appropriate size scale and batch-to-batch reproducible synthetic procedures of NPs with unique optical or magnetic properties is not sufficient to ensure biomedical application. For this, functionalization of the NPs with biomolecules is crucial in order to impart biological recognition and interaction skills.

Selecting the most adequate biofunctionalization strategy is no mean feat, since no universal methodologies exist to cover the wide variety of inorganic nanoparticles and biomolecules available for this purpose. A functionalization protocol that works well for one type of NP may not work for another, since they could be very different in terms of size, charge, surface area, colloidal stability, density and type of reactive groups, etc. Furthermore, biomolecules vary significantly in terms of size, chemical composition, 3D complexity and location of its biological active site. As discussed along this review, in absence of standard functionalization protocols, each particular case (nanoparticle + biomolecule) requires optimization. Thus, in addition to the development of "smart" multifunctionalization strategies, it is vital to focus on the synthesis of "smart" nanoparticles over the next decade. These NPs should be able to deliver a therapeutic agent based on environmental causes or remote stimulus and with the capability to temporarily adapt their size, shape, surface chemistry, wettability and adhesive properties to surrounding environments. These long-term goals would allow an overall impact on the medical field with significant advances in patient screening, monitoring, diagnosis, staging, and treatment.

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Nanomedicines for cancer therapy: state-of-the-art and limitations to pre-clinical studies that hinder future developments

***Charlene M. Dawidczyk*[1,2,3†], *Luisa M. Russell*[1,2,3†] *and Peter C. Searson*[1,2,3*]**

[1] *Institute for Nanobiotechnology, Johns Hopkins University, Baltimore, MD, USA*
[2] *Johns Hopkins Center of Cancer Nanotechnology Excellence, Johns Hopkins University, Baltimore, MD, USA*
[3] *Department of Materials Science and Engineering, Johns Hopkins University, Baltimore, MD, USA*

Edited by:
João Conde, Massachusetts Institute of Technology, USA

Reviewed by:
Yanli Zhao, Nanyang Technological University, Singapore
Fernando Novio, Institut Catalá de Nanociència i Nanotecnologia, Spain

****Correspondence:***
Peter C. Searson, Johns Hopkins University, 100 Croft Hall, 3400 North Charles Street, Baltimore, MD 21218, USA
e-mail: searson@jhu.edu

† These authors have contributed equally to this work.

The ability to efficiently deliver a drug or gene to a tumor site is dependent on a wide range of factors including circulation time, interactions with the mononuclear phagocyte system, extravasation from circulation at the tumor site, targeting strategy, release from the delivery vehicle, and uptake in cancer cells. Nanotechnology provides the possibility of creating delivery systems where the design constraints are decoupled, allowing new approaches for reducing the unwanted side effects of systemic delivery, increasing tumor accumulation, and improving efficacy. The physico-chemical properties of nanoparticle-based delivery platforms introduce additional complexity associated with pharmacokinetics, tumor accumulation, and biodistribution. To assess the impact of nanoparticle-based delivery systems, we first review the design strategies and pharmacokinetics of FDA-approved nanomedicines. Next we review nanomedicines under development, summarizing the range of nanoparticle platforms, strategies for targeting, and pharmacokinetics. We show how the lack of uniformity in preclinical trials prevents systematic comparison and hence limits advances in the field.

Keywords: drug delivery systems, nanoparticles, targeted therapy, pharmacokinetics, tumor accumulation

INTRODUCTION

Drug therapy often involves the use of small molecules such as alkylating agents (e.g., busulfan), anti-metabolites (e.g., gemcitabine), anti-microtubule agents (e.g., paclitaxel, vincristine), topoisomerase inhibitors (e.g., topotecan), and cytotoxic inhibitors (e.g., doxorubicin). These cytotoxic molecules kill highly proliferative cancer cells, but also other proliferative cells in bone marrow, the gastrointestinal (GI) tract, and hair follicles, leading to common side effects such as compromised immune system, inflammation and ulceration of the GI tract, and hair loss. Nanotechnology provides the possibility of creating delivery systems where the design constraints are decoupled, allowing new approaches for reducing the unwanted side effects of systemic delivery, increasing tumor accumulation, and improving efficacy.

The development of safe and efficient delivery systems is also important for advances in human gene therapy (Pack et al., 2005; Jones et al., 2013). A delivery system must transport a gene with high efficiency to target cells, with minimal toxicity and immune response. The main challenges for gene delivery are protecting the genetic material from degradation in circulation, avoiding degradation by enzymes in endosomes in the target cell, and escaping from endosomes to reach the nucleus or target compartment (Mintzer and Simanek, 2009; Zhang et al., 2012).

Key properties for drug and gene delivery systems are biocompatibility, stability in circulation, and increasing the fraction of the dose accumulating in the tumor. Drug toxicity can be reduced by encapsulating the free drug (e.g., liposomes) or by locally activating a pro-drug. Stability in circulation can be improved by developing strategies to minimize protein binding and evade the immune system. The efficiency of accumulation at a tumor site can be improved by active targeting of the delivery system or by increasing extravasation by the enhanced permeation and retention (EPR) effect.

The FDA-approved nanomedicines in clinical use have demonstrated the potential for increasing bioavailability, enhancing drug solubility, active targeting, and high drug loading (Dawidczyk et al., 2014). However, there remain many challenges in exploiting advances in nanotechnology and bioengineering to develop systems that will have significant impact on patient survival rates. The development of delivery systems remains largely empirical and the lack of standardization of pre-clinical studies is a barrier to establishing design rules for nanomedicines. While studies of complex systems with combined reporting/sensing functions along with drug or gene delivery may ultimately improve diagnosis and treatment, there are many fundamental issues that need to be addressed to establish the relationship between physico-chemical properties, pharmacokinetics, biodistribution, and survival rates.

Tumor uptake is modulated by the EPR effect (Jain and Stylianopoulos, 2010; Fang et al., 2011; Torchilin, 2011) and hence increasing the circulation time generally increases tumor accumulation. A common approach for increasing circulation time

is to provide a surface coating of polyethylene glycol (PEG). Whilst PEG coating increases circulation time and hence can increase tumor accumulation, it also inhibits uptake by tumor cells (Barenholz, 2012). Furthermore, PEG coating slows but does not prevent adsorption of opsonins that promote uptake by macrophages of the liver and spleen. Targeting molecules such as immunoglobulin-G (IgG) antibodies are opsonins and hence promote clearance by the mononuclear phagocyte system (MPS) (Walkey and Chan, 2012). The conjugation of folate to liposomes significantly increases their uptake by tumor-associated macrophages (Turk et al., 2004).

FDA-APPROVED NANOMEDICINES

There are currently six FDA-approved nanomedicines (**Table 1**): brentuximab vedotin and Trastuzumab emtansine, Doxil, DaunoXome, Marqibo, and Abraxane (Dawidczyk et al., 2014). Brentuximab vedotin and Trastuzumab emtansine are antibody-drug conjugates (ADCs), conceptually one of the simplest nanomedicines with an anticancer drug conjugated to a targeting molecule. Brentuximab targets the protein CD30, a glycosylated phosphoprotein expressed by B cells, including B-cell lymphomas, some leukemias, and melanoma cancer stem cells (Mullard, 2013; Sassoon and Blanc, 2013; Sievers and Senter, 2013). Trastuzumab targets the human epidermal growth factor receptor 2 (HER2) overexpressed in HER2 positive breast cancer (Lu et al., 2012; Verma et al., 2012). Monomethyl auristan E (MMAE) (Brentuximab vedotin) and mertansine (Trastuzumab emtansine) are too toxic to be used alone and hence coupling to a targeting antibody reduces toxic side effects. Several drug molecules are conjugated to each antibody via a valine-citrulline cleavable linker (Brentuximab vedotin) or covalent linkage (Trastuzumab emtansine) that is enzymatically degraded in endosomes following uptake. The small number of FDA-approved ADCs highlights the difficulty in translating relatively simple nanomedicines to the clinic.

Doxil, DaunoXome, and Marqibo are liposomal nanomedicines. Doxil is a pegylated liposome about 100 nm in diameter and encapsulating about 10,000 doxorubicin molecules (Barenholz, 2012). Encapsulation minimizes side effects, such as cardiotoxicity, associated with high doses of free doxorubicin. The concentration of doxorubicin in the liposomes is greater than the solubility limit and hence most of the drug is in the solid phase (Barenholz, 2012). The incorporation of cholesterol increases the bilayer cohesiveness and reduces leakage. These features minimize osmotic effects and contribute to stability, with more than 98% of the circulating drug remaining inside liposomes (Lasic et al., 1992; Gabizon et al., 1994, 2003). The polyethylene glycol coating is designed to give a long circulation half time and thereby increase tumor accumulation by the EPR effect (Immordino et al., 2006; Vllasaliu et al., 2014). While the

Table 1 | Summary of FDA-approved nanomedicines.

Platform	Class	Drug	d (nm)	Drug/carrier ratio	Key design feature(s)	Problem addressed
Brentuximab vedotin	ADC	Monomethyl auristan E	∼10	≤8	Valine-citrulline linker cleaved by cathepsin in endosomes	Monomethyl auristan E (MMAE) is too toxic to be used alone
Trastuzumab emtansine	ADC	Mertansine	∼10	≤8	Non-cleavable linker; release of drug by proteolytic degradation of antibody in endosomes	Mertansine is too toxic to be used alone
Doxil	Liposome	Doxorubicin	100	10,000–15,000	Lipid encapsulation for high drug/carrier ratio, polyethylene glycol coating to evade MPS, crystallization of drug in liposome minimizes escape during circulation	Drug toxicity and adverse cardiac side effects
DaunoXome	Liposome	Daunorubicin	50	∼10,000	No polyethylene glycol coating, targeted by MPS resulting in slow release into circulation	Drug toxicity and adverse cardiac side effects
Marqibo	Liposome	Vincristine	100	∼10,000	No polyethylene glycol coating, targeted by MPS resulting in slow release into circulation	Drug toxicity and adverse side effects
Abraxane	Protein carrier	Paclitaxel	130	>10,000	Non-specific binding of paclitaxel to albumin	Overcomes very low solubility of paclitaxel

mechanisms of uptake and release are not known, evidence suggests that the liposomes are taken up by endocytosis (Seynhaeve et al., 2013).

DaunoXome (Gill et al., 1996; Bellott et al., 2001; Lowis et al., 2006) and Marqibo (Bedikian et al., 2011; Silverman and Deitcher, 2013) are liposomal formulations of daunorubicin and vincristine, respectively. In contrast to Doxil, the design strategy for DaunoXome and Marqibo is to promote uptake by the MPS, providing a reservoir from which the free drug can enter circulation, similar to a slow infusion. This is achieved by not including pegylated lipids in the liposomes (Gill et al., 1996; Bellott et al., 2001; Silverman and Deitcher, 2013). DaunoXome is about 50 nm in diameter (Gill et al., 1996), and Marqibo is about 100 nm in diameter (Silverman and Deitcher, 2013).

Abraxane, or nab-paclitaxel (nanoparticle albumin bound), is lyophilized human serum albumin non-specifically bound to paclitaxel (Miele et al., 2009). Paclitaxel has very low solubility and is administered with the toxic non-ionic solvent Cremophor, which can lead to a wide range of allergic reactions. On injection, Abraxane particles dissociate into smaller albumin-paclitaxel complexes or unbound paclitaxel (Yardley, 2013). Since albumin is abundant in circulation, Abraxane provides a reservoir of a very low solubility drug in a non-toxic platform. The particles are about 130 nm in diameter and contain about 10,000 paclitaxel molecules (Miele et al., 2009).

The pharmacokinetics of these nanomedicines reflects their design (**Table 2**). Brentuximab vedotin and Trastuzumab emtansine both have moderate areas under the curve (AUCs), relatively low clearance, and long elimination half-times of 3–4 days (Younes et al., 2010; Lorusso et al., 2011; Girish et al., 2012; Lu et al., 2012; Bradley et al., 2013). Doxil has high AUC, low clearance rate, small distribution volume, and a long elimination half-time (Barenholz, 2012). These features are largely due to the polyethylene glycol coating that provides extended evasion of the MPS and minimizes distribution into peripheral tissues (Gabizon et al., 1994; Hubert et al., 2000; Lyass et al., 2000; Hong and Tseng, 2001; Hamilton et al., 2002). DaunoXome (Gill et al., 1996; Bellott et al., 2001; Lowis et al., 2006) and Marqibo (Bedikian et al., 2011; Silverman and Deitcher, 2013) have clearance rates about an order of magnitude larger than for the ADCs and Doxil, low distribution volumes, and short elimination half-times on the order of 10 h. The larger AUC associated with DaunoXome is related to the larger dose range compared to Marqibo. Abraxane has a fast clearance rate, about two orders of magnitudes larger than DaunoXome and Marqibo, large distribution volume, and elimination half-time similar to DaunoXome and Marqibo (Sparreboom et al., 2005; Ando et al., 2012). The pharmacokinetics for Abraxane are similar to free paclitaxel and the other free drugs: low AUC, high clearance rate, high distribution volume, and short elimination half-time.

Overall it is evident that antibody drug conjugates or liposomes with a pegylated surface have long elimination half-times, typically of 3–4 days. Increasing elimination half-times is expected to increase tumor accumulation via the EPR effect. However, increased tumor accumulation does not necessarily imply improved efficacy since processes such as transport, uptake, drug release, and delivery to the appropriate cellular compartment are all downstream of extravasation by the EPR effect.

NANOPARTICLE PLATFORMS, TARGETING MOIETIES

NANOPARTICLE PLATFORMS

The development of a broad range of nanoparticle platforms with the ability to tune size, composition, and functionality has provided a significant resource for nanomedicine (**Table 3**) (Niemeyer, 2001; Duncan, 2006; Cho et al., 2008; Greco and Vicent, 2009; Yu et al., 2013). Nanoparticle platforms can be broadly categorized as organic, inorganic, and hybrid.

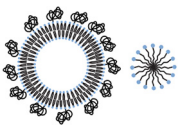
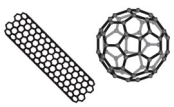
Organic nanoparticles have been widely explored for decades, yielding a large variety of materials, formulations, imaging modalities, cargo, and targets for cancer therapy.

Table 2 | Summary of pharmacokinetics for FDA-approved nanomedicines and corresponding free drugs from human clinical trials.

Drug	Dose mg^2/m	AUC (mg h/L)	CL (L/h)	V_d(L)	$t_{1/2}$(h)	References
Brentuximab vedotin	90–110	3.2–4.9	0.071–0.075	8.2–10.2	106–144	Younes et al., 2010; Bradley et al., 2013
Trastuzumab emtansine	10–160	0.6–28	0.023–0.070	1.7–3.5	31–98	Lorusso et al., 2011; Girish et al., 2012; Lu et al., 2012
Doxil	25–80	600–4900	0.023–0.045	2.1–6.4	42–90	Gabizon et al., 1994; Hubert et al., 2000; Lyass et al., 2000; Hong and Tseng, 2001; Hamilton et al., 2002
DaunoXome	10–190	17–1700	0.40–0.94	2.9–4.1	2.8–8.3	Gill et al., 1996; Bellott et al., 2001; Lowis et al., 2006
Marqibo	2.0–2.25	5–15	0.36–0.38	2.6–2.9	9.6–12	Bedikian et al., 2011; Silverman and Deitcher, 2013
Abraxane	150–300	4–10	31–67	900–1700	11–26	Sparreboom et al., 2005; Ando et al., 2012
Doxorubicin	15–72	0.5–3.8	25–72	250–1800	9–29	Erttmann et al., 1988; Jacquet et al., 1990; Piscitelli et al., 1993; Gabizon et al., 1994
Daunorubicin	40–120	1–19	110–150	200–450	9–24	Bellott et al., 2001; Krogh-Madsen et al., 2012
Paclitaxel	170–330	6–40	15–50	160–530	7.2–7.6	Sparreboom et al., 2005

In most cases, data represent the range of mean or median values for obtained from different doses. For unit conversion we used an average body surface area of 1.7 m^2, an average body weight of 60 kg, and a blood volume of 5 L.

Table 3 | Summary of nanoparticle platforms for nanomedicine.

	Particle type	Composition/Structure	Properties	Applications
	Polymer	e.g., PLGA, glycerol, chitosan, DNA; monomers, copolymers, hydrogels	Some biodegradable	Drug delivery; passive release (diffusion), controlled release (triggered)
	Dendrimer	PAMAM, etc.	Low polydispersity, cargo, biocompatible	Drug delivery
	Lipid	Liposomes, micelles	Can carry hydrophobic cargo, biocompatible, typically 50–500 nm	Drug delivery
	Quantum dots	CdSe, CuInSe, CdTe, etc.	Broad excitation, no photobleaching, tunable emission, typically 5–100 nm	Optical imaging
	Gold	Spheres, rods, or shells	Biocompatibility, typically 5–100 nm	Hyperthermia therapy, drug delivery
	Silica	Spheres, shells, mesoporous	Biocompatibility	Contrast agents, drug delivery (encapsulation)
	Magnetic	Iron oxide or cobalt-based; spheres, aggregates in dextran or silica	Superparamagnetic, ferromagnetic (small remanence to minimize aggregation), superferromagnetic (~10 nm), paramagnetic	Contrast agents (MRI), hyperthermia therapy
	Carbon-based	Carbon nanotubes, buckyballs, graphene	Biocompatible	Drug delivery

Organic polymer systems include synthetic polymers [e.g., polyethyleneimine (PEI), polyethylene glycol] (Knop et al., 2010; Nicolas et al., 2013), synthetic hydrogels (e.g., polyacrylamide) (Ando et al., 2012; Liechty and Peppas, 2012), natural polymers (e.g., chitosan, hyaluronic acid, alginate, gelatin) (Ando et al., 2012) and hydrolytically or enzymatically degradable polymers (e.g., collagen, polylactic acid, polycaprolactone) (Balogh et al., 2007). Combinations of components and/or monomer units and incorporation of other building blocks such as DNA contribute to the flexibility of polymer-based nanoparticle platforms. These systems can be passively loaded with a cargo, or a cargo can be incorporated to allow triggered release (Davis et al., 2008). Particles such as block copolymers, liposomes, and dendrimers can provide a reservoir for large amounts of cargo. Block copolymers combine the attributes of two or more monomer units allowing further functionality (Duncan, 2006; Greco and Vicent, 2009). Lipid-based nanoparticles include micelles, liposomes, or water oil emulsions. Dendrimers are hyperbranched synthetic polymers for which biodistribution, size, and multifunctionality can be tuned with a very low degree of polydispersity (Cho et al., 2008). Proteins (e.g., albumin) (Fuchs and Coester, 2010) and viruses (Steinmetz, 2010) have also been extensively studied for drug and gene delivery.

Inorganic nanoparticles provide advantages in function and properties not possible with organic nanoparticle platforms, although this is often at the expense of biocompatibility. Examples of materials include semiconductors (quantum dots) (Gao et al., 2004; Medintz et al., 2005; Michalet et al., 2005; Park et al., 2011; Chen et al., 2012a; Petryayeva et al., 2013), silica (Vanblaaderen and Vrij, 1992; Giri et al., 2005, 2007; Burns et al., 2006), gold (Boisselier and Astruc, 2009; Arvizo et al., 2010), magnetic materials (Arruebo et al., 2007; Banerjee et al., 2010; Haun et al., 2010), and carbon-based materials (Prato et al., 2008; Jain, 2012). Semiconductor nanoparticles, or quantum dots, have a narrow and tunable emission spectrum, a broad excitation spectrum, and do not photobleach. These characteristics are attractive for optical imaging, however, many quantum dots are synthesized from heavy metal elements and hence toxicity is a concern. Silicon dioxide (silica), the most widely used oxide, is a versatile material that is relatively inert. Silica can be used to encapsulate other materials or cargoes and the surface can be conjugated using silane chemistry. Silica can be synthesized with nanometer scale pores (mesoporous silica) that can be used to hold other cargoes. Of the metallic materials, gold is widely used for biological applications as it is easy to synthesize, can be functionalized using thiol chemistry, and is relatively inert. Many of the noble metals absorb

electromagnetic radiation in the visible range of the spectrum (plasmon absorbance), and have been explored for hyperthermia therapy. Gold nanoparticles conjugated with PEG and tumor necrosis factor alpha (TNFα are being developed for targeted cancer therapy (Libutti et al., 2010).

Ferromagnetic materials, such as iron oxide (magnetite, Fe_3O_4), iron, cobalt, and nickel offer an additional degree of freedom in the synthesis of nanoparticles for nanomedicine (Arruebo et al., 2007). Very small ferromagnetic nanoparticles (typically < 10 nm) have no intrinsic magnetization in the absence of a magnetic field, and hence do not aggregate in colloidal suspension. These superparamagnetic nanoparticles can be manipulated in an external field providing a simple method for spatial manipulation and washing. Magnetic nanoparticles, such as superparamagnetic iron oxide (SPIO) nanoparticles have been used for magnetic resonance imaging (MRI) and hyperthermia therapy (Yu et al., 2013).

Carbon-based nanoparticles have exploited the small size and unique properties of buckyballs, carbon nanotubes, and grapheme (Yu et al., 2013). Combinations of organic and inorganic materials, taking advantage of specific materials and structures have also been widely explored in multifunctional nanoparticle platforms.

Hybrid nanoparticles with organic and inorganic components or associated combinations of inorganic nanostructures provide further opportunities for introducing multiple functionalities. These systems can exploit the biocompatibility of organic nanoparticles, while still retaining the stability and function of inorganic nanoparticles. Inorganic nanoparticle conjugates allow for multimodal imaging and theranostic applications. Examples include constructs such as liposomes filled with magnetic nanoparticles (Sailor and Park, 2012), coordination polymer nanoparticles (Novio et al., 2013), and metal-organic frameworks (Horcajada et al., 2012).

TARGETING MOIETIES (ANTIBODIES, APTAMERS, SMALL MOLECULES, ETC.)

Active targeting of a nanoparticle is a way to minimize uptake in normal tissue and increase accumulation in a tumor. Strategies for active targeting of tumors usually involve targeting surface membrane proteins that are upregulated in cancer cells (Huynh et al., 2010; Hanahan and Weinberg, 2011). While this strategy is widely used, tumor cell populations are extremely heterogeneous and expression levels can vary significantly. Targeting molecules are typically antibodies (Dill et al., 1994; Arruebo et al., 2009; Chames et al., 2009), antibody fragments (Holliger and Hudson, 2005), aptamers (Keefe et al., 2010; Hu and Zhang, 2013), or small molecules (**Figure 1**).

Accumulation of a delivery system at a tumor site by the EPR effect is dependent in part on the concentration in the circulation. Processes such as clearance by the MPS or uptake in normal tissue decrease the concentration in circulation and hence decrease the accumulation in the tumor. Active targeting can provide an additional sink for a nanoparticle platform since expression of target molecules is usually differential in that the target is highly expressed in tumor cells but expressed at low levels in other cell types in the vascular system. Since the surface area of the vasculature is much larger than the tumor, active binding in normal tissue can be significant, even for targets that are expressed at relatively low levels (Jain, 2005). Furthermore, targeting moieties may themselves be targets for receptors on phagocytic cells, as described above.

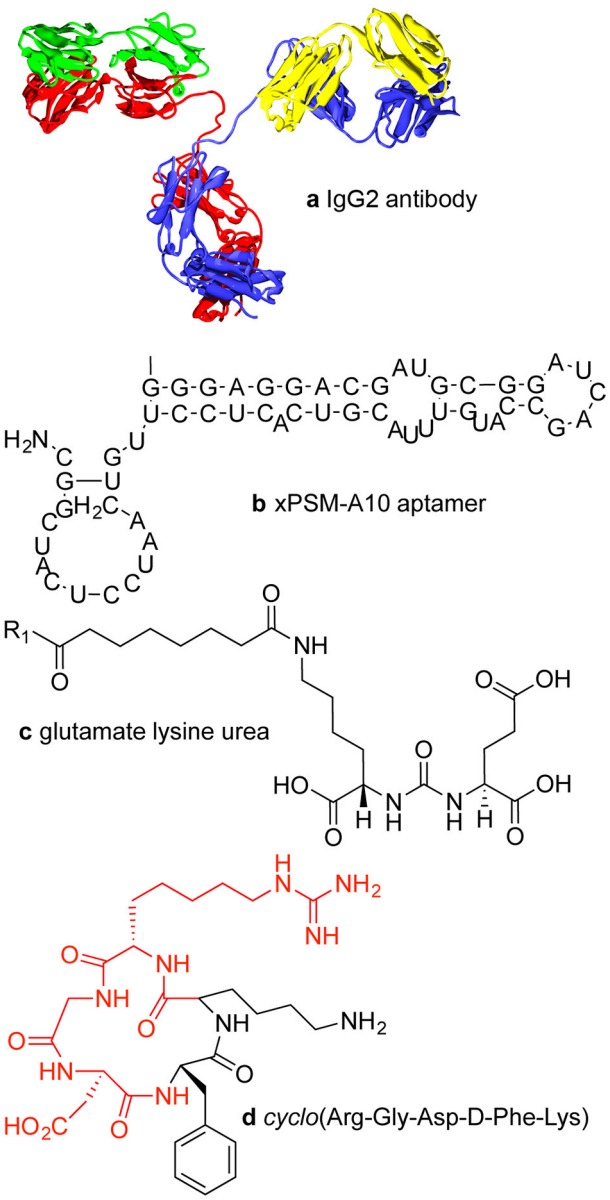


FIGURE 1 | Examples of targeting molecules. (A) Antibodies are typically around 150 kDa or about 15 × 5 nm with two antigen binding sites. **(B)** xPSM-A10 is a 18.5 kDa aptamer with a binding affinity of about 10^{-8} M^{-1} for the extracellular portion of the prostate-specific membrane antigen (PSMA) (Lupold et al., 2002). **(C)** The glutamate lysine urea small molecule targets PSMA (473 Da) (Banerjee et al., 2008). **(D)** The RGD peptide sequence (604 Da) binds to cell surface integrins, upregulated in many tumor types.

Antibodies

Monoclonal IgG antibodies (mAbs) are widely used for protein recognition and targeting since they have two epitope binding

sites, high selectivity, and high binding affinity (Chames et al., 2009). Antibodies are the largest of the targeting ligands, approximately 150 kDa or about 15 nm long and about 5 nm in diameter. The binding (dissociation) constants for antibody—antigen interactions vary over a wide range from 10^{-6} to 10^{-9} M, but can be as high as 10^{-12} M for high affinity antibodies (Dill et al., 1994). For targeting applications, the Fc region of the antibody can be a disadvantage if it is accessible to Fc receptors on macrophages, which can lead to increased accumulation in the liver and spleen (Allen, 2002).

Antibody fragments

Antigen binding sites represent only a small part of the overall size of antibodies. $F(ab')_2$ fragments retain both antigen binding sites of the antibody coupled by disulfide linkages. Cleavage of the disulfide bond under reducing conditions yields two Fab′ fragments with sulfhydryl groups that can be used for coupling to the targeting platform. Single chain variable fragments maintain only the variable regions (variable light chain and variable heavy chain) of one arm of an antibody.

Aptamers

Aptamers are folded single strand oligonucleotides, 25–100 nucleotides in length (8–25 kDa) that bind to molecular targets (Tuerk and Gold, 1990; Keefe et al., 2010). High throughput screening methods can be used for rapid selection of aptamers for specific targets (Bunka and Stockley, 2006). Macugen, approved for use in the treatment of macular degeneration in 2004, is currently the only FDA approved aptamer (Adamis et al., 2006).

Small molecules

Small molecules for targeting include peptides, growth factors, carbohydrates, ureas, and receptor ligands (Weissleder et al., 2005). Specific examples include folic acid, transferrin, and the RGD peptide sequence. Folic acid (441 Da) is recognized by the folic acid receptor and is expressed in normal epithelial cells but is overexpressed in many cancer types, especially ovarian, brain, and lung cancers (Kamen and Smith, 2004; Hilgenbrink and Low, 2005; Parker et al., 2005; Chames et al., 2009; Muller and Schibli, 2013; Naumann et al., 2013). Folic acid is essential for amino acid synthesis and hence for cell survival and proliferation, and has a high affinity ($K_d < 10^{-9}$ M) (Hartmann et al., 2007). Transferrin is a chelating protein that regulates the supply of iron into cells via receptor-mediated endocytosis (Kresse et al., 1998). The transferrin receptor is expressed at low levels in most normal tissues but is overexpressed in many tumor types (Daniels et al., 2012). The RGD (Arg-Gly-Asp) peptide is a target for integrins (e.g., $\alpha_v\beta_3$) on the cell surface (Ruoslahti, 1996; Hynes, 2002). RGD is a component of the extracellular matrix protein fibronectin and promotes cell adhesion and regulates cell migration, growth, and proliferation (Ruoslahti, 1996; Hynes, 2002). A cyclic peptide containing the RGD sequence is widely used for targeting to integrins (Haubner et al., 1996). The upregulation of integrins is promoted by angiogenic factors in several cancer types (Dechantsreiter et al., 1999; Hosotani et al., 2002; Furger et al., 2003; Sheldrake and Patterson, 2009).

TUMOR ACCUMULATION AND TARGETING EFFICIENCY

In preclinical studies the efficacy of a drug is often determined from the time dependence of tumor size or from the fraction of animals that survive after a candidate therapy. These parameters are particularly useful in assessing the potential therapeutic benefit of a new delivery system but integrate many factors. An additional parameter that is important in assessing the potential efficacy of delivery systems is the tumor accumulation or targeting efficiency—the fraction of an intravenously administered dose that accumulates in a tumor (%ID). Despite the importance of this parameter, very few measurements are reported in the literature.

We have reviewed 40 pre-clinical studies of delivery systems employing passive targeting (Supplementary Table S1), and 34 pre-clinical studies employing active targeting (Supplementary Table S2). Only studies reporting quantitative results of tumor accumulation were selected. Analysis of these pre-clinical studies highlights the need for guidelines to improve the overall impact of research in this field. Despite the importance of pharmacokinetics and tumor accumulation in assessing the efficiency of delivery systems, very few preclinical studies report quantitative results that can be used to develop design rules for nanomedicines.

PASSIVE TARGETING

Delivery systems used in pre-clinical studies exploiting passive targeting include liposomes (Harrington et al., 2000; Wang et al., 2006; Soundararajan et al., 2009; Zheng et al., 2009; Huang et al., 2011; Chen et al., 2012a; Coimbra et al., 2012; Hsu et al., 2012; Mahakian et al., 2014) (Kheirolomoom et al., 2010), micelles (Yokoyama et al., 1999; Le Garrec et al., 2002; Kawano et al., 2006; Reddy et al., 2006; Rijcken et al., 2007; Kim et al., 2008; Hoang et al., 2009; Shiraishi et al., 2009; Blanco et al., 2010; Sumitani et al., 2011; Wang and Gartel, 2011; Zhao et al., 2012; Miller et al., 2013; Zhu et al., 2013), gold nanoparticles (Hainfeld et al., 2006; Von Maltzahn et al., 2009; Puvanakrishnan et al., 2012), iron oxide nanoparticles (Ujiie et al., 2011), silica nanoparticles (Chen et al., 2012b; Di Pasqua et al., 2012), carbon-based nanostructures (Liu et al., 2011; Robinson et al., 2012; Rong et al., 2014), quantum dots (Sun et al., 2014), and hybrid nanomaterials (Balogh et al., 2007; Tinkov et al., 2010; Yang et al., 2012) (Paraskar et al., 2012) (Ohno et al., 2013) (Supplementary Table S1).

Of the 40 pre-clinical studies, only a few (4/40) reported tumor accumulation as %ID, while the remainder reported normalized accumulation as %ID/g or %ID/cc. The tumor accumulation varies over a wide range from 0.1 to 35%ID/g at 24 h post-injection. Passive delivery systems are generally pegylated and have sizes in the range from 2 to 200 nm. However, there are no clear trends in terms of identifying physico-chemical parameters that influence the pharmacokinetics or tumor accumulation. Although pegylation is generally assumed to increase circulation time and hence increase tumor accumulation, there is no consistent difference in tumor accumulation between pegylated and non-pegylated delivery systems.

Similarly, there is no obvious dependence on the size or shape of the delivery system. For example, the tumor accumulation of pegylated liposomes around 100 nm in diameter in three

studies varied from 0.4 to 11%ID/g (Soundararajan et al., 2009; Kheirolomoom et al., 2010; Hsu et al., 2012). The large variation is likely due to the differences in xenograft cell line, tumor size, and dose. Similarly, tumor accumulation in two pre-clinical studies of 30 nm diameter micelles with different polymer formulations were 1.5%ID/g (Yokoyama et al., 1999) and 9.5%ID/g (Blanco et al., 2010). These two studies used different models (orthotopic vs. xenograft), tumor cell line (A549 vs. C26), tumor size (200 vs. 100 mm^3), and injected dose (30–50 mg/kg vs. 10 mg/kg). These differences in experimental design limit the ability to compare the two different micelle formulations. These examples highlight the difficulty in comparing pre-clinical trials due to the variability in experimental design.

ACTIVE TARGETING

Targeted delivery systems used in quantitative pre-clinical studies include silica (Benezra et al., 2011; Tang et al., 2012; Chen et al., 2013), gold (Melancon et al., 2008; Lu et al., 2009, 2010; Chanda et al., 2010; Choi et al., 2010; Morales-Avila et al., 2011; Chattopadhyay et al., 2012), liposomes (Iyer et al., 2011; Helbok et al., 2012; Petersen et al., 2012), micelles (Hu et al., 2008; Penate Medina et al., 2011; Zhang et al., 2011b; Fonge et al., 2012; Helbok et al., 2012) (Rossin et al., 2005; Khemtong et al., 2009; Zhan et al., 2010; Poon et al., 2011; Zhang et al., 2011a; Xiao et al., 2012), iron oxide (Natarajan et al., 2008; Kumar et al., 2010; Yang et al., 2011), graphene (Hong et al., 2012; Cornelissen et al., 2013; Shi et al., 2013), gadolinium (Oyewumi et al., 2004), polymer nanocarriers (Kunjachan et al., 2014), nanoemulsions (Ohguchi et al., 2008), quantum dots (Gao et al., 2010), and hybrid (Cheng et al., 2014) (Supplementary Table S2). Similar to passive targeting, few studies (3/34) report %ID rather than %ID/g. The most common targeting ligands are antibody based (9/34 studies), the RGD peptide sequence (10/34), and folate (5/34). Targeting efficiencies obtained using RGD peptides, folate, antibodies, and antibody fragments are typically between 1 and 15%ID/g (Supplementary Table S2).

Assessing the efficiency of a targeting ligand in increasing tumor accumulation is complicated by the different control experiments used in these studies. The contribution of passive targeting was assessed by measuring tumor accumulation of the delivery system without attachment of the targeting ligand (20/34), with attachment of a non-specific ligand (2/34), pre-injection with a blocking molecule or treatment (10/34), or with a xenograft formed from a cell line that did not express the target molecule (2/34). Several studies (4/34) did not report a control experiment. Each control experiment has advantages and disadvantages. For example, removing a targeting ligand from a delivery system may alter the physico-chemical properties and hence change the pharmacokinetics. As described in more detail below, xenografts formed from different cell lines may have significantly different vascularization and hence the rate of extravasation to the tumor site by the EPR effect may be significantly different. Pre-injection with a blocking molecule may not completely prevent binding to the target molecule or may reduce binding in normal tissue. To account for these potential complications, a few studies (3/34) used multiple controls.

Of the 30 pre-clinical studies that reported control experiments, 33% (10/30) showed less than a two-fold increase in targeting compared to the control, and 50% (15/30) showed an increase in tumor accumulation of more than 2%ID/g with the targeting ligand. For example, a tumor accumulation of 9%ID was reported for SPIONS with anti-ChL6 2 days post-injection compared to 1% without the targeting antibody (Natarajan et al., 2008). A tumor accumulation of 7 ± 1%ID was reported for gadolinium nanoparticles with a folate targeting ligand, and 9 ± 4%ID in the control with no targeting ligand (Oyewumi et al., 2004). While active targeting of a delivery system to a tumor site has the potential to reduce unwanted side effects, these studies highlight the difficulties in assessing targeting efficiency due to the large differences in experimental design and the range of controls used to assess the contribution of passive targeting.

TUMOR ACCUMULATION

In general, the uptake of a delivery system in a tumor tends to increase post-injection but then decreases at longer times (Supplementary Table S1 and S2). For example, tumor accumulation of radiolabeled liposomes increased to 11.3%ID/g over the first 24 h, then decreased to 6.1%ID/g after 72 h (Hsu et al., 2012). Tumor accumulation of self-activating quantum dots increased to 13%ID/g over the first 24 h, but decreased to 11%ID/g after 42 h (Sun et al., 2014). Similarly, tumor accumulation of pegylated micelles with a gelatinase binding peptide was reported to increase to almost 18% ID/g over the first 6 h, but decreased to 2% ID/g after 24 h (Penate Medina et al., 2011). Tumor accumulation of gold nanoparticles with the RGD peptide increased to 3.65% ID/g over the first hour followed by a decrease by almost half to 1.94% ID/g 24 h post-injection (Morales-Avila et al., 2011). The details of the time dependence of tumor accumulation are important in understanding the pharmacokinetics, the EPR effect, and the limitations to accumulating a drug at the tumor site. In many studies, an insufficient number of time points precludes detailed analysis of pharmacokinetics and tumor accumulation.

The cell line used in forming a xenograft can have significant influence on tumor accumulation and efficacy. In the 74 quantitative pre-clinical trials reviewed here, 35 different cell types were used to form xenografts. The most common cell lines were the 4T1 murine breast cancer cell line (10/71) and the C26 colon carcinoma cell line (10/71), both of which form highly vascularized tumors. Tumor accumulation of micelles with the RGD peptide was 6%ID/g in a mouse model with a C26 xenograft and 3%ID/g with a less leaky BxPC3 xenograft (Kunjachan et al., 2014), highlighting the need for standardization of cell lines.

Tumor size can have a significant influence on tumor accumulation. For example, a study using radiolabeled liposomes compared targeting efficiency among tumors of different sizes using the KB cell line (Harrington et al., 2000). The tumor accumulation for small tumors ($\leq$0.1 g) was around 15%ID/g, whereas for larger tumors ($\geq$1 g) was only 3%ID/g.

GUIDELINES FOR PRE-CLINICAL STUDIES OF DELIVERY SYSTEMS

While the physico-chemical properties of delivery systems are expected to exert a significant influence on pharmacokinetics,

tumor accumulation, and biodistribution, there are numerous problems in comparing pre-clinical studies. In particular, differences in cell line and tumor size, dose, lack of good pharmacokinetics data, and differences in reporting make meta-analysis extremely difficult and are a limitation to progress in the field (**Table 4**). Similarly, physico-chemical properties of the delivery system such as size, surface properties (i.e., pegylation), zeta potential, targeting ligand density, and stability in blood or serum at physiological temperature are not uniformly reported.

For example, results are usually reported as percent of initial administered dose per gram of tumor (%ID/g), which is only useful if the tumor mass is also reported. For example, a tumor accumulation of 10%ID/g is 10% of the initial dose for a 1 g tumor but 1% of the initial dose for a 0.1 g tumor. These differences are significant in terms of the efficiency of delivery and minimizing unwanted side effects in normal tissue. In some cases tumor characteristics such as tumor diameter or approximate tumor volume are reported, however, these parameters can only be used to estimate the absolute percentage of the initial dose.

Table 4 | Summary of limitations to pre-clinical studies of nanomedicines that hinder broad assessment of design rules.

Problem	Solution
Total tumor accumulation (%ID) is not always reported Inconsistent reporting of tumor size/weight	Report tumor accumulation as %ID (and %ID/g) Report tumor size/weight
Inconsistent reporting of dose	Report dose as total number of nanoparticles injected Along with other parameters such as drug loading, drug concentration (and/or drug amount), and activity of dose (gamma counter)
Inconsistent reporting of physico-chemical properties	Report standard physico-chemical properties (e.g., size, zeta potential, surface coating, stability under physiological conditions)
Tumor accumulation reported at different time points	Report tumor accumulation at standard time points (e.g., 1 and 24 h post-injection). Detailed pharmacokinetics (concentration in blood and tumor) at multiple time points is preferred
Variation in tumor characteristics (type, size, vascularization, etc.)	Standardize tumor type and size (e.g., C26 or 4T1; 1 cm diameter) More difficult for active targeting depending on target molecule
Variation in controls used in active targeting	Report control studies for delivery system with no targeting ligand and any differences in physico-chemical properties. Report other control studies as necessary
Variation in animal models (mouse, rat, etc.) and differences in drug concentration compared to humans	Use mouse xenograft model for initial pre-clinical studies
Different detection methods used to assess tumor accumulation	Perform validation using other method(s)

Mouse models are widely used for research studies of disease progression and the development of new therapies (Frese and Tuveson, 2007). Rat and rabbit models are also commonly used for pre-clinical studies. Standard tumor models include subcutaneous xenografts of human cell lines or explants, orthotopic xenografts, and genetically engineered mouse models (Frese and Tuveson, 2007; Chen et al., 2012a). While these models are invaluable for pre-clinical studies, differences in physiology can lead to differences in circulation and tumor accumulation compared to humans (Steichen et al., 2012).

Xenografts represent a relatively straightforward model to study the pharmacokinetics, tumor accumulation, and biodistribution of a nanomedicine, however, tumor characteristics vary considerably with cell line and size (Harrington et al., 2000; Jain and Stylianopoulos, 2010). The density and vascularization of tumors of similar size can also vary significantly. Highly invasive cell lines often form more highly vascularized tumors, for example xenografts of colon cancer cell lines have vasculature that is much more leaky than pancreatic cancer cell lines. Therefore, tumor uptake by the EPR effect is expected to be strongly dependent on the cell line used. Establishing a standard cell line and tumor size for xenografts would greatly enhance comparison of pre-clinical trials of delivery systems (**Table 4**). While this is feasible for passive delivery systems, active targeting often requires the use of specific cell lines that overexpress a particular biomarker.

Tumor accumulation is usually measured using a gamma counter, positron emission tomography (PET), or inductively coupled plasma mass spectroscopy (ICP-MS). The methods using a gamma counter or PET require that a suitable radiolabel is conjugated to the drug delivery platform. With a gamma counter, the radioactivity of the resected tumor is measured and compared to the radioactivity of the dose. To determine the tumor accumulation from PET scans, reconstructed 3D regions of interest are drawn around the tumor. The activity per unit mass can then be determined after correcting for decay and tissue density. An alternative to using a radiolabel to measure the tumor accumulation is to use ICP-MS to determine the amount of one or more elemental components in the delivery system and to compare to the initial dose. However, this method requires a component of the delivery system to be distinguishable from biological matter. In most pre-clinical studies only one of the methods is used to determine pharmacokinetics and tumor accumulation and hence there is no independent verification.

Tumor accumulation is expected to be dependent on the dose and time post-injection, and hence time-course studies at different doses are important for full characterization. In many cases, tumor accumulation is determined only at one or two time points therefore limiting analysis of the pharmacokinetics which is crucial for developing design rules.

SUMMARY

Nanoparticle-based delivery systems provide new opportunities to overcome the limitations associated with traditional drug therapy and to achieve both therapeutic and diagnostic functions in the same platform. The efficiency of drug or gene delivery to a tumor site is dependent on the physico-chemical properties of the delivery platform and a range of physiologically imposed design constraints including clearance by the mononuclear phagocyte system and extravasation from circulation at the tumor site by the enhanced permeability and retention effect.

The lack of uniformity in pre-clinical trials of nanoparticle-based delivery systems has prevented systematic comparison of these studies and has been an impediment to developing design rules for new systems or specific applications. Of the large number of pre-clinical trials, surprisingly few report quantitative data on parameters that would be useful in developing design rules for nanomedicines. The poor experimental design and variability of experimental conditions also contribute to slow development of the field and the lack of clinical impact. We highlight some of the problems with pre-clinical trials nanoparticle-based delivery systems and suggest some solutions to increase the impact of individual studies.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fchem.2014.00069/abstract

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Gold nanoparticles and their alternatives for radiation therapy enhancement

Daniel R. Cooper, Devesh Bekah and Jay L. Nadeau*

Department of Biomedical Engineering, McGill University, Montreal, QC, Canada

Edited by:
Jesús M. De La Fuente, Universidad de Zaragoza, Spain

Reviewed by:
Pedro Viana Baptista, Universidade Nova de Lisba, Portugal
Wolfgang Parak, Universität Marburg, Germany

***Correspondence:**
Jay L. Nadeau, Department of Biomedical Engineering, McGill University, 316 Lyman Duff Building, 3775 University Street, Montreal, QC H3A 2B4, Canada
e-mail: jay.nadeau@mcgill.ca

Radiation therapy is one of the most commonly used treatments for cancer. The dose of delivered ionizing radiation can be amplified by the presence of high-Z materials via an enhancement of the photoelectric effect; the most widely studied material is gold (atomic number 79). However, a large amount is needed to obtain a significant dose enhancement, presenting a challenge for delivery. In order to make this technique of broader applicability, the gold must be targeted, or alternative formulations developed that do not rely solely on the photoelectric effect. One possible approach is to excite scintillating nanoparticles with ionizing radiation, and then exploit energy transfer between these particles and attached dyes in a manner analogous to photodynamic therapy (PDT). Doped rare-earth halides and semiconductor quantum dots have been investigated for this purpose. However, although the spectrum of emitted light after radiation excitation is usually similar to that seen with light excitation, the yield is not. Measurement of scintillation yields is challenging, and in many cases has been done only for bulk materials, with little understanding of how the principles translate to the nanoscale. Another alternative is to use local heating using gold or iron, followed by application of ionizing radiation. Hyperthermia pre-sensitizes the tumors, leading to an improved response. Another approach is to use chemotherapeutic drugs that can radiosensitize tumors. Drugs may be attached to high-Z nanoparticles or encapsulated. This article discusses each of these techniques, giving an overview of the current state of nanoparticle-assisted radiation therapy and future directions.

Keywords: nanoparticle, scintillator, radiation therapy, photodynamic therapy, photosensitizer, radiosensitizer

INTRODUCTION AND BACKGROUND

Radiation therapy (XRT) is a critical component of the modern approach to curative and adjuvant treatment of cancers. XRT controls the growth of cancerous cells by bombardment with ionizing radiation, causing DNA damage by direct ionization or through generation of free radicals by ionization of water or oxygen molecules. Sufficient damage to DNA in this fashion can arrest cell growth and prevent metastasis. The primary drawback is collateral damage: there is little distinction in absorption between healthy and malignant tissues, and thus doses must be limited in order to mitigate unwanted damage to the tumor surroundings. External beam radiotherapy (EBRT) utilizes X-ray beams produced by orthovoltage units, or linear accelerators that may be spatially oriented, with beams shaped using multileaf collimators in order to maximize the specificity for the target. Distinct energy ranges are available for different EBRT targets: 40–100 kV (kilovoltage or "superficial" X-rays) for skin cancers or other exposed structures; as well as 100–300 kV (orthovoltage) and 4–25 MV (megavoltage or "deep" X-rays) for sub-surface tumors. Techniques such as 3-dimensional conformal and intensity-modulated radiation therapies have vastly improved the targeting capabilities of external beam therapy, but naturally there is still a strong desire to be able to further reduce the doses required for effective treatment. The SI derived unit for absorbed dose is the gray (Gy), equivalent to one joule of energy deposited by ionizing radiation per kilogram of matter ($1\,\text{Gy} = 1\,\text{J/kg} = 1\,\text{m}^2/\text{s}^2$).

Brachytherapy, or internal radiotherapy, utilizes a radioactive source to provide a steady or pulsed dose of radiation to a small tissue volume. It is typically used for cervical, prostate, breast and skin cancers. Radioactive sources include ^{125}I and^{103}Pd, which produce γ rays of ~20–35 keV, ^{192}Ir (γ rays, 300–610 keV), ^{137}Cs (γ rays, 662 keV), ^{60}Co (γ rays, 1.17 and 1.33 MeV), ^{198}Au (γ rays, 410–1009 keV), ^{226}Ra (γ rays, 190–2430 keV), and ^{106}Ru which decays primarily through β^- emission at 3.54 MeV. Seeds of the listed materials can provide doses of up to 12 Gy/hour (high dose rate or HDR brachytherapy), though typical low dose rate (LDR) treatments amount to around 65 Gy over 5–6 days.

Heavy elements can be potent radiosensitizers (Kobayashi et al., 2010). It has been demonstrated that platinum-containing DNA-crosslinking drugs such as Cisplatin can enhance the effects of ionizing radiation through the "high Z effect," or what has come to be known as Auger therapy. Heavy elements have significantly higher photoelectric cross-sections than soft tissue for sub-MeV energies, approximated for "X-ray energies" by the equation:

$$\sigma_{pe} \propto \frac{Z^n}{E^3}$$

where σ_{pe} is the cross-section, $E = h\nu$ is the photon energy, Z is the atomic number, and n varies between 4 and 5 depending

on the value of *E*. The photoelectric effect dominates below the electron rest energy of 511 keV, beyond which inelastic Compton scattering becomes more prevalent. As the photon energy decreases, it is no longer able to eject inner-shell electrons, producing the characteristic sawtooth pattern with K, L, and M edge structures. When ionized by X-ray or γ ray energy, mid- to high-Z elements (roughly Br and up) can produce a cascade of low-energy Auger electrons that can locally enhance the effective radiation dose (Kobayashi et al., 2010). Dense inorganic nanoparticles can also provide radiation dose enhancement that depends upon the composition and size of the particles, uptake of particles into cells, and the energy of the applied radiation.

GNRT

Au nanoparticles have been under investigation for several years as possible agents for selective amplification of radiation dose in tumors, a concept called "gold nanoparticle-assisted radiation therapy" or GNRT (McMahon et al., 2008; Brun et al., 2009; Cho et al., 2009; Rahman et al., 2009; Van den Heuvel et al., 2010; Leung et al., 2011; Zhang et al., 2012). Reviews of this work can be found in Jelveh and Chithrani (2011), Butterworth et al. (2012), Jain et al. (2012), Babaei and Ganjalikhani (2014), Su et al. (2014).

The earliest studies used bulk or micro-sized gold to enhance radiation dose. Although this could be effective *in vitro* at a range of energies, micron-sized particles are not taken up well *in vivo*, even after intratumoral injection (Herold et al., 2000). Later experiments focused on Au nanoparticles or nanoclusters (1.9 nm diameter). When injected intravenously, these ultrasmall particles rapidly accumulated in cancer tissue, with 2.7 g Au/kg body weight resulting in 7 mg Au/g in tumor almost immediately after injection. Irradiation was performed about 60 s after injection, and with typical 250 kVp X-ray therapy, 1-year survival was 86% (compared to 20% with X-rays alone and 0% with gold alone) (Hainfeld et al., 2004). This result was followed by theoretical and experimental papers examining the mechanism of Au nanoparticle action as well as attempting to optimize Au particle concentration, size, and the energy and dose of applied X-rays (Cho et al., 2009; Zhang et al., 2009; Van den Heuvel et al., 2010; Leung et al., 2011).

IMPROVING GNRT BY TARGETING

A significant problem with ultrasmall, nontargeted nanoparticles is rapid excretion by the kidneys. The amount of Au needed in early studies (>2 g Au/kg body weight) represents a very large amount of Au for human use. This is impractical, costly, and may cause toxicity. Achieving therapeutic levels in tumor with less delivered total Au is needed. In addition, irradiation in the mouse studies was performed immediately after particle injection. This is not practical in the clinic and may not work well in humans. Particles with longer circulation times, which can be delivered in multiple doses, are desirable for clinical applications. Optimizing the size, surface chemistry, and targeting of the Au nanoparticles may improve circulation times and accumulation in specific tumors.

The increased metabolic rate of tumors relative to normal tissue results in a high demand for glucose. Several studies have used

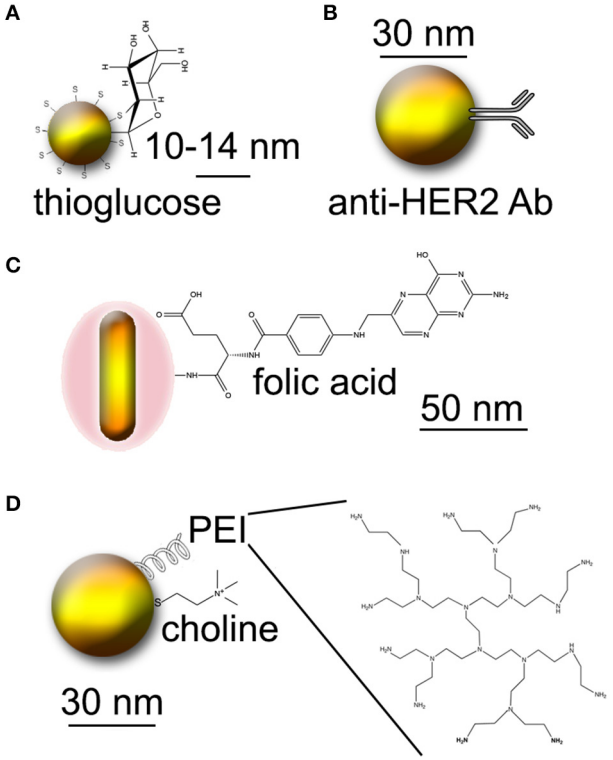

FIGURE 1 | Approaches to creating tumor-targeted Au nanoparticles. Molecules not to scale. **(A)** Thioglucose-conjugated Au nanoparticles. **(B)** Au nanoparticles conjugated to Herceptin (anti-HER2 antibody). **(C)** Au nanorods conjugated to folic acid. **(D)** PEI-coated Au nanoparticles conjugated to choline.

thioglucose-conjugated Au nanoparticles (**Figure 1A**) in order to increase uptake by cancer cells. One study using ∼14 nm Au demonstrated significantly increased uptake of thioglucose-conjugated particles by an ovarian cancer cell line after 8–96 h of incubation (Geng et al., 2011). A significant increase in inhibition was seen in the presence of 5 nM particles using 90 kVp or 6 MV X-rays; dose enhancement was significant relative to control beginning at 5 Gy and extending to 20 Gy, where all cells were inhibited even in the absence of particles. Another study compared cysteamine and thioglucose-coated 15 nM Au nanoparticles in breast cancer and normal breast cell lines (Kong et al., 2008b). Cysteamine-coated particles were taken up 3- to 4-fold more efficiently than glucose-coated particles. However, when applied to cells at concentrations that led to similar intracellular Au concentrations, glucose-coated particles led to increased radiosensitization relative to cysteamine-capped particles. Interestingly, radiosensitization by Au was not seen in a nonmalignant breast cell line, although the cells grew at the same rate as the cancer cells and took up an equal number of particles. The ability of ^{167}Cs and ^{60}Co sources to inhibit the cancer cells was also demonstrated in this paper.

The use of larger Au particles (57 nm and 84 nm) coated with thioglucose has been studied in another report (Song et al., 2013). These particles were taken up in equal numbers by HeLa cells.

Surprisingly, unconjugated particles showed a greater radiosensitizing effect than thioglucose-conjugated particles, which the authors attributed to possible absorbance of singlet oxygen by the thioglucose shell.

Another study used the humanized anti-HER2 antibody Trastuzumab (Herceptin), PEGylated and conjugated to 30 nm Au particles, for delivery to MDA-MB-361 breast cancer (Chattopadhyay et al., 2013) (**Figure 1B**). Both *in vitro* and *in vivo* studies were performed. *In vitro*, an effective dose enhancement factor of 1.6 was seen in the presence of 2.4 mg/mL particles using 100 kVp X-rays. Delivery to MDA-MB-361 xenografts was done intratumorally, with ∼0.8 mg total Au used (4.8 mg/g tumor). 11 Gy of 100 kVp image guided X-ray irradiation was performed 24 h after injection. This subtoxic dose led to a 46% reduction in tumor size relative to irradiation alone, with no damage to normal tissue or systemic toxicity.

Folic acid is another nutrient for which the need is increased in cancer cells. Conjugation to folate has been used for a wide variety of targeting applications for cancer and inflammatory diseases; a review may be found here (Low et al., 2008). Intra-operative tumor imaging using folate targeting has recently moved to the clinic for ovarian cancer (van Dam et al., 2011). In terms of GNRT, one study reported the use of silica-modified Au nanorods (∼50 nm long) conjugated to folate (Huang et al., 2011) (**Figure 1C**). The rods were taken up by MGC803 human gastric carcinoma cells. 6 Gy of X-irradiation led to a 60% decrease in cell viability in the presence of 12.5 μM rods relative to cells without Au. The study also demonstrated uptake of the rods by MGC803 xenografts in nude mice, with contrast sufficient for X-ray imaging. No radiation experiments on animals were reported.

Cancers are also often distinguished by a lower pH than healthy cells due to hypoxia and resulting anaerobic metabolism within tumors. The pH-sensitive pHLIP peptide was used in one study to target Au nanoparticles to mice bearing HeLa tumors (Yao et al., 2013). Although radiation was not performed, accumulation of Au in tumors sufficient for radiotherapy enhancement was demonstrated, with the stated goal of using the construct for this purpose.

Prostate cancer is an excellent target for nanoparticle-enhanced radiation, since it is often treated by brachytherapy and is accessible to intratumoral injection. Choline is a ubiquitous molecule in all cells for which overactivity of processing enzymes (choline kinase) has been found in prostate tumors. One study reported development of polyethylene imine (PEI)- and choline-conjugated Au nanoparticles for the purpose of targeting prostate cancer for GNRT (Razzak et al., 2013) (**Figure 1D**). While no radiation experiments were performed in this study, favorable pharmacokinetics were shown in mice.

These studies illustrate that targeted GNRT remains an area requiring substantial further investigation. While one or more targeting agents may be conjugated to Au nanoparticles, and while these may improve delivery *in vitro* and even *in vivo*, it is not fully established whether these formulations improve tumor response to radiation therapy. The possibility that an organic shell can absorb reactive oxygen species deserves further inquiry. The size of the nanoparticles used, the density of targeting ligands, and the delivery method (IV, intratumoral, concentration, timing) all need to be optimized. The good news is that many of these formulations use FDA-approved ingredients, some of which are currently in the clinic for imaging. Optimization in animal studies should thus lead to rapid clinical translation.

IMPROVING GNRT BY ADDITION OF PHOTOTHERMAL THERAPY

Hyperthermia therapy is a minimally invasive treatment in which the temperature is increased locally (up to 44°C) to kill malignant cells. Even though hyperthermia can kill cells on its own, it is more often used in combination with other treatments such as radiotherapy or chemotherapy (Wust et al., 2002); such combinations are in clinical trials (Vernon et al., 1996; van der Zee et al., 2000; Zagar et al., 2010). An increase in nuclear damage is one of the mechanisms through which cells are radiosensitized after hyperthermia (Wust et al., 2002; Kampinga, 2006). In addition, the higher temperature causes dilation of the blood vessels, increasing oxygenation of the tumor (Griffin et al., 1996; Song et al., 2009). Since oxygen is a potent radiosensitizer, it can increase the damage to the tumor through generation of free radicals.

Methods to locally heat the tumor region include high intensity focused ultrasound (HIFU), microwave heating, magnetic hyperthermia, and photothermal therapy. In photothermal therapy, a light source (usually infrared) is used to deliver heat to the tumor. Such approaches are difficult to target, but delivery of nanomedicines to the tumor could improve the local heating profile. Most studies have looked at gold nanoparticles and nanorods for this purpose, because exposure of Au nanoparticles to IR light causes a local temperature increase due to surface plasmon resonance (El-Sayed et al., 2006; Huang et al., 2006; Gobin et al., 2007; Hainfeld et al., 2010; Verma et al., 2014). By modifying the size and shape of these nanoparticles, the resonance peak can be tuned to different wavelengths in the IR.

Delivery of gold followed by heating and ionizing radiation has proved to be a promising approach in pre-clinical studies. Gold nanoshells with a 120 nm silica core and a 12–15 nm shell were used in one study (Diagaradjane et al., 2008) to treat a murine xenograft model of human colorectal cancer. Localized hyperthermic treatment followed 5 min later by a 10 Gy X-ray dose were given 20–24 h after IV delivery of the nanoshells. The tumor volume doubling time was significantly greater for the treated mice. Two mechanisms were identified as contributing to the treatment's efficacy: an increase in perfusion resulting in a decrease in tumor hypoxia, and vascular collapse in the tumor due to accumulation of nanoparticles around the blood vessels. Another study confirmed these results using similar gold nanoshells in two murine breast cancer models (Atkinson et al., 2010).

Another study used gold nanorods modified with silica and conjugated to folic acid (Huang et al., 2011) to test the effects of photothermal and radiation therapy on MGC803 gastric cancer cells. The two treatments were tested separately and not combined. For the radiation treatment, a 6 MeV source was used to deliver doses of up to 10 Gy. Cell survival with radiation decreased in a concentration-dependent fashion with nanoparticle addition; the particles were non-toxic in the absence of radiation.

Photothermal therapy consisted of 3 min of irradiation with a 30 mW, 808 nm laser source. Apoptosis was seen after treatment in the presence of the gold particles.

A recent study (Hainfeld et al., 2010) calculated the radiation dose required to control 50% of tumors (TCD50) in a mouse squamous cell carcinoma model. Gold nanoparticles were delivered intratumorally, and 24 h later the tumor was heated to 48°C for 5 min at 1.5 W/cm^2 followed by X-ray irradiation at 100 kVp (7.5 Gy/min). TCD50 was reduced from 55 Gy to less than 15 Gy.

These studies illustrate one of the biggest problems of the approach, which is the need for simultaneous delivery of heating and radiation, which poses logistic problems in the clinic (Wust et al., 2002). Other drawbacks include a lack of specificity and the difficulty of heating deep tumors.

ALTERNATIVES TO GOLD: BISMUTH AND IRON

Alternatives to Au are being sought that are more effective and/or less costly. Bismuth (Bi, $Z = 83$) and platinum (Pt, $Z = 78$) have been shown in at least one theoretical study to yield a dose enhancement factor higher than Au, with Bi being the highest. Dose enhancement is predicted to increase with decreasing nanoparticle size, because the smaller nanoparticles accumulate closer to the nucleus, where they can cause the greatest damage. The dose enhancement is also expected to be greater when the average energy is close to the K-edge of the element (Ngwa et al., 2010; Hossain and Su, 2012). A radiochromic dosimeter was used in another study to experimentally measure the dose enhancement of bismuth oxide (Bi_2O_3) nanoparticles. Using a 100 kV X-ray source and an irradiation dose of 10 Gy, the radiation dose in a water-equivalent matrix doped with 0.5 mM of 50 nm Bi_2O_3 nanoparticles was >80% higher than in the control compartment (Alqathami et al., 2013). Another study (Zhang et al., 2014) looked at the dark toxicity, biodistribution, and radiation effects of bismuth selenide (Bi_2Se_3) nanoplatelets in cell lines and mice. The platelets were not significantly toxic to either cells or mice, with over 93% of the Bi cleared from the body 90 days after treatment. Significant radiation dose enhancement was observed after irradiation doses of up to 8 Gy.

Gadolinium (Gd, $Z = 64$) represents another alternative to gold nanoparticles. In addition to having a relatively high atomic number, Gd is already routinely used as a contrast agent in MRI. Gd_2O_3 core nanoparticles encapsulated in a polysiloxane shell have shown potential as an image guided radiotherapeutic tool in a gliosarcoma rat model (Le Duc et al., 2011). Accumulation of the nanoparticles in the tumor after saphenous vein injection was demonstrated using MRI, and the tumor-bearing rats were treated with microbeam radiation therapy, with a significant increase in survival in the nanoparticle-treated group. Another study using a rat brain tumor model confirmed that ultra-small Gd-based nanoparticles accumulate in brain tumors after IV injection (Miladi et al., 2013).

Magnetic particles such as iron oxide may also be used for combined hyperthermia and radiation. By using an alternating magnetic field to excite magnetic nanoparticles, local temperature increases can be achieved. The advantages of iron oxide include low toxicity, ease of synthesis, and the ability to perform image guidance using MRI. Dextran-coated iron oxide has been shown to reduce tumor growth in a syngeneic mouse breast cancer model when hyperthermia and radiation were combined (Giustini et al., 2011).

Several studies have looked at radiosensitization properties of iron oxide nanoparticles. Using 6 MeV X-rays on a human prostate carcinoma cell line (DU145), 1 mg/ml of Fe_3O_4 nanoparticles resulted in a dose enhancement factor of approximately 1.2 (Khoei et al., 2014). Another study suggested that superparamagnetic iron oxide nanoparticles (SPIONs) can radiosensitize tumor cells by catalyzing ROS formation. Uncoated, citrate-coated, or malate-coated SPIONs were added to MCF-7, 3T3, and Caco-2 cells. Uncoated SPIONS caused dark toxicity, with no increase in ROS upon 1 or 3 Gy irradiation. In contrast, coated SPIONS were non-toxic in the absence of radiation, but resulted an increase of up to 300% in the fluorescence intensity of the ROS reporter dichlorofluorescein diacetate (DCF-DA) (Klein et al., 2014).

SCINTILLATING NANOPARTICLES FOR RADIATION/PHOTODYNAMIC "HYBRID" THERAPY

INTRODUCTION AND CONCEPT

A 2006 study proposed a new approach to nanoparticle-based therapies aiming to combine and enhance the effects of radiation therapy and photodynamic therapy (PDT) through the use of scintillating nanoparticles conjugated to photosensitizer molecules (Chen and Zhang, 2006). The concept is simple: attach a dye used for PDT to a nanoparticle that emits light when excited by therapeutic radiation (scintillates). If the scintillation emission overlaps the absorbance spectrum of the dye, the dye will generate singlet oxygen as it does with light-excited PDT (**Figure 2**). Many conventional photosensitizers are based on naturally occurring porphyrin, chlorin, and bacteriochlorin structures comprised of highly conjugated heterocyclic macrocycles (**Figure 3A**). These molecules have a strong absorbance peak in the UV to blue range (Soret band) as well as numerous weaker peaks in the visible (**Figure 3B**).

This idea has attracted significant attention over the past few years (Cheng and Lo, 2011) because it promises to combine the tissue penetration depth of radiation with the efficacy and benign side effect profile of PDT. PDT results in less damage to normal tissue than does radiation therapy; does not induce scarring; may be repeated multiple times; and may spark immune responses that help destroy the tumor. However, because of the limited tissue penetration depth of visible and even near-IR light, this therapy is restricted to only the most superficial cancers, such as non-melanoma skin cancer and bladder cancer.

The challenge is to develop stable, nontoxic nanoscintillators that may be delivered to cells. Several varieties of doped insulator and semiconductor nanoparticles have been proposed to fill this role. While scintillation has been demonstrated for CdSe/ZnS quantum dots (Létant and Wang, 2006), they have poor radiation hardness and degrade rapidly under γ ray exposure (Withers et al., 2008). As the toxicity of these materials is also primarily related to their chemical degradation, alternatives are necessary. The development of such alternatives is mostly in early stages. Although many of the approaches to surface chemistry and targeting that have been used for gold could be applied to these other

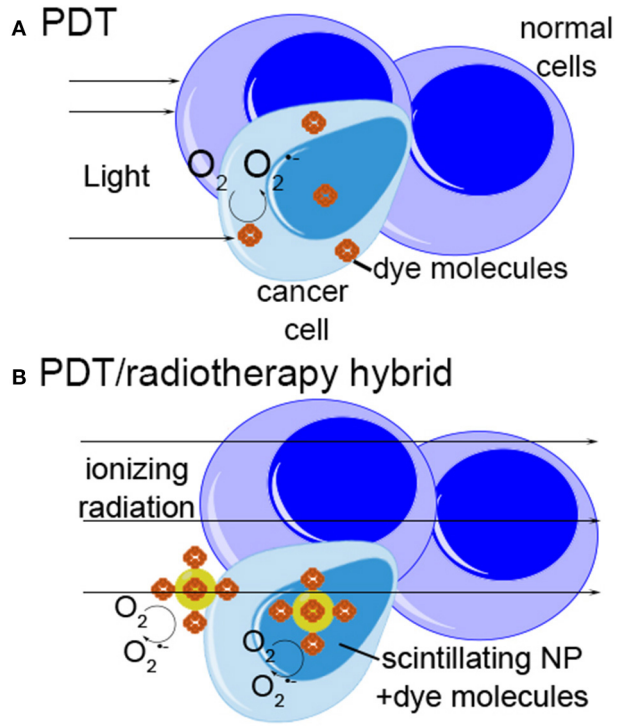

FIGURE 2 | Schematic of photodynamic therapy and radiotherapy-photodynamic therapy "hybrid" approach. (A) In photodynamic therapy, photosensitizer dye molecules collect preferentially in malignant or inflamed tissue. Light is used to excite the dye, generating reactive oxygen species which lead to cell killing. **(B)** In the "hybrid" approach, ionizing radiation is used to excite scintillating nanoparticles, which may be located deep within tissue. The nanoparticles transfer energy to attached photosensitizer molecules, generating ROS and killing cells by the same mechanism as photodynamic therapy.

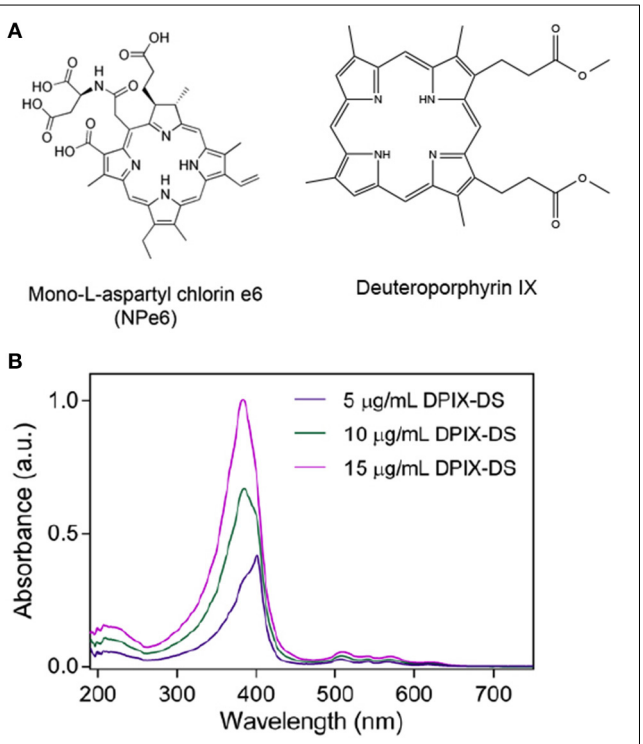

FIGURE 3 | Photosensitizers. (A) Typical photosensitizer structures: mono-L-aspartyl chlorin e6 (Talaporfin sodium), a PDT drug that can be isolated from algae or green plants (approved in Japan and in Phase III trials in the U.S.); deuteroporphyrin IX, a candidate photosensitizer with several possible derivatives. **(B)** Absorbance spectra of different concentrations of deuterophorphyrin IX disulfonic acid (DPIX-DS). Note the strength of the Soret band (UV-blue) compared to the peaks in the redder regions.

materials, this has not yet been attempted. Some of the materials also show specific chemical challenges as we will discuss in Section Biocompatibility of lanthanide-based materials.

SCINTILLATION

Scintillation, or radioluminescence (RL), is the process whereby a material, referred to as a scintillator, produces light upon interaction with ionizing radiation. Inorganic nanoparticles (NPs) doped with lanthanides present an attractive, radiostable alternative to quantum dots for scintillation.

Introduction to lanthanide luminescence

Lanthanides are well known for the luminescence of their trivalent cations, which emit primarily through phosphorescence resulting from electronic transitions within the *4f* shell (Bünzli and Eliseeva, 2010). Because these transitions are "forbidden" by Laporte's parity selection rule (formally prohibiting electric dipole transitions between states that conserve parity), they have low absorption cross-sections and their photoluminescence is commonly sensitized by Ce^{3+} (for downconversion, with Tb^{3+} acceptor) or Yb^{3+} (for upconversion, with Tm^{3+}, Er^{3+}, and Ho^{3+} acceptors), though more complex combinations of lanthanides are certainly possible. The efficiency of both processes benefits from a low phonon energy host, though is of increasing importance for lower energy transitions. In the case of upconverting NPs, hexagonal phase (β phase) $NaYF_4$ or isostructural $NaGdF_4$ are generally the preferred host materials.

The mechanism of cerium luminescence is distinct from most other lanthanides. Neutral cerium has a $[Xe]4f^15d^16s^2$ electronic configuration; in solution or in solid hosts, the +3 or +4 oxidation states are the most common. Only the +3 state is luminescent, though the +4 state also has important implications for redox activity. In the +3 state, the 6*s* and 5*d* electrons are lost, leaving one optically active electron in the shielded 4*f* shell. Fluorescence ($\Delta S = 0$) arises from parity-allowed, high oscillator strength 4*f*-5*d* transitions. Because the 5*d* orbitals are external, these transitions are sensitive to the crystal field, and vary in energy across a substantial range depending on the host material (Dorenbos, 2000).

Cerium-doped lanthanum fluoride ($Ce_xLa_{1-x}F_3$) shows luminescence in the UV-blue (corresponding well to the Soret band) and so is a likely candidate for useful energy transfer to photosensitizers.

Mechanisms of scintillation

RL mechanisms of bulk $Ce_xLa_{1-x}F_3$ crystals were elucidated in the late 80 s and early-to-mid 90 s as candidates for radiation

detection purposes (Moses and Derenzo, 1989, 1990; Wojtowicz et al., 1992, 1994; Lempicki et al., 1993; Moses et al., 1994; Rodnyi et al., 1995). Though the scintillation was found to be significantly faster than commonly used scintillators at the time (BGO, CsI:Tl, NaI:Tl) on a per-photon basis, the overall light output was found to be unexpectedly weak, with variable luminescence that was significantly dependent on the quality of the crystal and the presence of defects. This variability precluded their use as reliable detectors for the most part, at least compared to other options being developed concurrently, such as $PbWO_4$.

The general process of activator-based scintillation occurs in three steps: first, conversion of absorbed ionizing radiation energy into electronic-lattice excitations (electron-hole pairs and/or excitons), followed by transfer of the excitation energy to the emitting centers and then luminescence. The overall scintillation efficiency is given by the product of the individual efficiencies:

$$\eta = \beta SQ, \quad 0 \leq \eta, \beta, S, Q \leq 1$$

where β, the efficiency of the conversion process, encompasses the fraction of absorbed energy lost to optical phonons, S is the efficiency of the transfer process, and Q is the luminescence quantum yield of the emitting center. The overall light output L (in photons/MeV) is given by:

$$L = n_{e-h}\eta = \frac{10^6}{2.3E_g}\beta SQ$$

where n_{e-h} is the number of *e-h* pairs or excitons that are generated per MeV of absorbed radiation, discounting losses to optical phonons, and $E_g$is the band gap of the host (in eV). The factor of 2.3 is related to the derived minimum incident photon energy required to generate a single *e-h* pair (Robbins, 1980), $\xi_{\min} = 2.3E_g$, and so $n_{e-h} = E/2.3E_g$ where E is the energy of the incident photon, in this case 1 MeV $= 10^6$ eV.

Low phonon energy hosts such as LaF_3 tend toward higher values of β, while the transfer process S is relatively inefficient compared to pentaphosphate or orthophosphate hosts (Lempicki et al., 1993). The β and S mechanisms of $Ce_xLa_{1-x}F_3$ were determined to consist of three distinct processes that have different relative contributions depending on the value of x: (i) direct excitation of Ce^{3+} by X-rays or secondary electrons, (ii) ionization of Ce^{3+} followed by electron capture and formation of bound excitons, or (iii) energy transfer to Ce^{3+} from lattice excitations of the bulk matrix. At lower concentrations of Ce^{3+}, up to $x \sim 0.5$, mechanism (iii) dominates the scintillation response. At higher doping levels, mechanism (i) is predominant, accounting for a large fraction of the light output in CeF_3. It has recently been demonstrated that co-doping single crystals of YPO_4:Ce^{3+} with Pr^{3+}, which act as electron traps, can improve scintillation efficiency by minimizing the influence of defects as well as mitigating the effects of damage caused by prolonged irradiation (Moretti et al., 2014).

Nanoscintillators

A number of reports have investigated the scintillation response of $Ce_xLa_{1-x}F_3$ nanocomposites, where small NPs (~10 nm in diameter) are cast into oleic acid or polymer matrices with consistencies ranging from liquid to waxy. In initial studies, nanocomposites exhibited photopeaks for ^{137}Cs, ^{241}Am, and ^{57}Co irradiation (McKigney et al., 2007a,b). Most recently, a modest scintillation response (compared to a BC-400 polyvinyltoluene detector) has been shown for 25% NP-loaded composites exposed to several sources: ^{22}Na (3.22 μCi), ^{60}Co (3.78 μCi), ^{137}Cs (31.9 μCi), ^{241}Am (9.09 μCi), and ^{252}Cf (5.03 μCi) (Guss et al., 2013). For radiation detection purposes, fast lifetimes are typically preferred, whereas for bioconjugates, short lifetimes may preclude efficient energy transfer if it is outcompeted by luminescence or quenching processes.

While the scintillation of cerium in simple fluoride or phosphate hosts is well studied, it is just one of a number of possible scintillation mechanisms. In the late 2000 s, a number of reports were released discussing the possibilities and limitations for nanoscintillators in a broad sense, including the demonstration of a few crucial nanoscale phenomena (Klassen et al., 2008, 2009; Dujardin et al., 2010; Kortov, 2010). Several research groups are now engaged in the development of a wider variety of nanoscintillators, either through adaptation of known scintillating materials to the nanoscale, or through the creation of novel compositions. Many of these are based on luminescent "activator" dopants, including lanthanides (Ce^{3+}, Pr^{3+}, Tb^{3+}, or $Eu^{2/3+}$). RL spectra have been published for a number of fluoride nanoscintillators, including powdered LaF_3:Eu (~4.4 nm), BaF_2:Ce (~10 nm), and CaF_2:Eu (~18 nm) NPs under excitation by a 40 kV Bullet X-ray tube and CaF_2:Eu^{3+} excited by a 1 μCi ^{241}Am source ($E_\alpha = 5.5$ MeV, $E_\gamma = 60$ keV) (Jacobsohn et al., 2011). The authors suggest that in such doped ionic crystals, where the diffusion length of *e-h* pairs may be up to 100 nm, it is conceivable that scintillation yields may be limited by the physical dimensions of the NPs or by the total number of activators. The same group has also compared the effects of undoped LaF_3 shell thickness on the photoluminescence *vs.* RL of LaF_3:Eu NPs (Jacobsohn et al., 2010). The undoped shells act as a passivating barrier that is transparent to both optical excitation and emission, and PL efficiency was found to increase in a roughly linear fashion as a function of overall NP size as additional shells were added. With X-ray excitation, the shells were found to increase RL efficiency up to a shell volume of roughly twice the core volume, beyond which the light yield decreased with additional shell thickness. This was attributed to the increased undoped volume decreasing the probability of radiative recombination within the Eu-doped core volume, and suggesting that the diffusion length of carriers in LaF_3 to be relatively short.

Indeed, the luminescence of core-only activator-based nanoscintillators has been found to be size-dependent in some cases. One study demonstrated a considerable broadening of Eu^{3+} emission lines in progressively smaller Gd_2O_3 NP hosts as compared to bulk crystals, attributed to increasing crystal field fluctuations in the smaller NPs (Dujardin et al., 2010). A number of physical mechanisms potentially influencing nanoscintillators are described in the report, including structural effects, surface effects, quantum confinement, and dielectric confinement. Also shown was a significant difference in the RL spectra of bulk *vs.* nanoscale CeF_3 samples. Intriguing scintillation behavior

from $LuBO_3$:Ce nanocrystals has been reported, with a considerable dependence on the NC dimensions (Klassen et al., 2008, 2009). NC grain sizes were controlled by altering annealing temperatures, and scintillation yields were found to increase dramatically for NCs ∼95 nm in diameter, with roughly three times the intensity of NCs either 25 nm larger or smaller. This is in contrast to LuF_3:Ce NPs in the same size range, which exhibited a monotonic size dependence.

Synthesis techniques and post-synthesis processing affect the size, crystallinity, and dopant distribution of nanostructures. The role of post-synthesis annealing on NCs was recently investigated with $LaPO_4$:Eu and $LaPO_4$:Pr (Malyy et al., 2013), as well as $LuPO_4$:Ce (Vistovskyy et al., 2014). In the case of $LaPO_4$:Ln, annealing was used to increase the size of the NCs, also resulting in a change of lattice symmetry above ∼500°C. The subsequent effects on excitation processes over the range of 4–40 eV are described in some detail. Across the energy range investigated, the distinct mechanisms include intracenter excitation, charge transfer excitation, exciton or e-h pair creation, electronic excitation multiplication ($E > 2\ E_g$), or combinations (**Figure 4**), and different sensitivities were shown for the two activators—the first stage of Eu^{3+} recombination involving electron capture, in contrast to hole capture by Pr^{3+}. With $LuPO_4$:Ce, substantial differences in the low energy (4–25 eV) VUV excitation spectrum and PL and RL decay kinetics were observed after the NCs were annealed for 2 h at 1200°C (*vs.* at 800°C, 300°C, or unannealed), corresponding to an increase in the crystallite size from 3 nm to 35 nm. The increased size resulted in well-defined PL emission components, dramatically enhanced band-to-band excitations above ∼8.7 eV, and the elimination of the slow RL decay component ascribed to surface defects. Importantly, the RL intensity for 35 nm NCs was found to be ∼100× stronger than for smaller (<12 nm) NCs, whereas the PL intensity of both types was comparable.

The synthesis and characterization of a number of Pr^{3+} and Ce^{3+}-activated garnet, silicate and oxide nanoscintillators have recently been reported, with an emphasis on their use for combined XRT/PDT, in particular their emission in the 300–400 nm range (Jung et al., 2014). The RL properties of powdered nanocrystalline samples prepared through combustion synthesis and annealing at 1200°C were compared with single/microcrystalline samples of similar compositions. The general composition $(Y_{1-x}Pr_x)_3Al_5O_{12}$ [or yttrium aluminum garnet (YAG):Pr, with an average diameter of 80 nm] was found to have the highest scintillation yield of the nanoscintillators tested under 50 keV excitation, though with a different activator concentration dependence than single crystal samples: quenching was observed for $x > 1\%$, compared to $x = 0.16$–0.65% reported for single crystals. Somewhat surprisingly, only YAG:Pr NCs with $x = 0.75$, 1, and 1.5% had greater emission intensity than $Bi_4Ge_3O_{12}$ (BGO) NCs, in stark contrast to single crystals, where BGO had the lowest relative intensity of the compositions investigated. Indeed, because the RL behavior of NCs is dependent on activator concentration quenching, which is in turn dependent on the NC composition, size and crystallinity, it was suggested that the properties of different preparations will likely have to be evaluated individually rather than predicted by bulk trends. The introduction of the article also provides an inclusive overview of recent progress in nanoscintillator research for biomedical applications.

Nanoscintillators that do not emit through specific activator ions are referred to as self-activated (SA), with luminescence arising from core-valence transitions, self-trapped excitons,

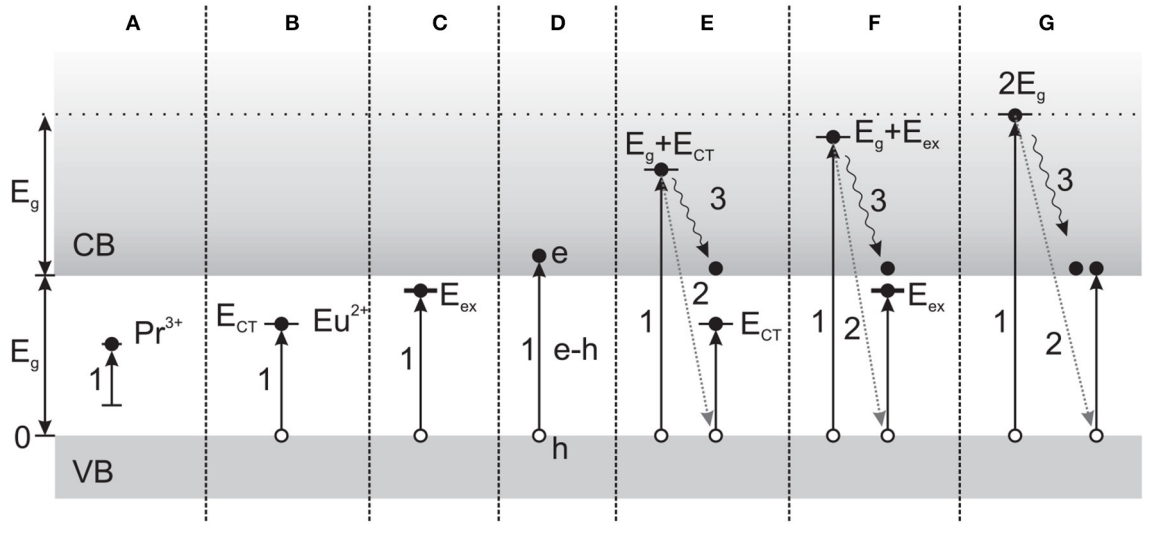

FIGURE 4 | Mechanisms of scintillation in Pr^{3+} or Eu^{3+}-doped $LaPO_4$, depending on excitation energy. (A) Intracenter (direct) excitation of Ln activators. **(B)** Excitation by charge transfer from O^{2-} to Eu^{3+}. **(C)** Direct exciton formation. **(D)** Creation of e-h pairs. **(E)** Excitation multiplication, with secondary excitation as in **(B)**. **(F)** Excitation multiplication, with secondary excitation as in **(C)**. **(G)** Photons with $E > E_g$ can result in excitation multiplication involving the creation of secondary e-h pairs. Arrows: (1) Transition due to photon absorption. (2) Energy exchange due to inelastic scattering on valence band electrons, and (3) relaxation of primary electrons. (Reprinted with permission from Malyy et al., 2013).

charge-transfer emissions or other mechanisms. YAG, BaF_2, and Y_2O_3 are among those that have been adapted to the nanoscale, but have not yet been investigated to a large extent as SA nanoscintillators. These compositions are also routinely doped with other activators, resulting in various effects on their intrinsic luminescence.

BIOCOMPATIBILITY OF LANTHANIDE-BASED MATERIALS

Preparation of LnNP bioconjugates (covalent attachment of organic molecules of interest to the NP surface ligands) appears infrequently in the literature. The principles of bioconjugation are similar to those for QDs, Au, or other NPs, with some distinct stability and solubility concerns (Cao et al., 2012; Jiang et al., 2012). Ligand-exchanged and silicated LnNPs typically present primary amine functionalities which provide some additional versatility over carboxyl groups. Amines provide a number of conjugation routes, including routine reactions with amine, isothiocyanate, carboxyl, hydroxyl, and thiol functional groups on a molecule of interest. One study reported conjugates of phosphorylethanolamine (PEA)-stabilized Eu^{3+} and Ce^{3+}/Tb^{3+}-doped LaF_3 by reacting the free amine of the ligand with activated biotin-PEG or mPEG NHS esters, demonstrating a successful strategy for attachment of molecules through amide bond formation. The use of these conjugates was restricted to borate buffer. Biotin conjugates have also been prepared with CeF_3:Tb NPs silanized using TEOS/aminopropyltriethoxysilane (APTES) (Kong et al., 2007, 2008a) and PEA-stabilized Ln^{3+}-doped zirconia (Liu et al., 2012).

SCINTILLATING NANOPARTICLE INTERACTIONS WITH DYES AND PHOTOSENSITIZERS (PSs)

When nanoparticles are conjugated to PS molecules and irradiated with ionizing radiation, singlet oxygen yield will depend upon scintillation yield and energy transfer efficiency. Neither of these parameters has been widely reported in the literature. However, a good number of studies have investigated lanthanide-dye charge transfer using light excitation, and a few studies have looked at singlet oxygen generation.

Lanthanide energy and charge transfer (ET and CT) have been extensively studied for lanthanide chelates and organic dye pairs (Selvin, 1996, 2002), and more recently in LnNPs, though most efforts have focused on sensitization of $4f$-$4f$ luminescence by Ce^{3+}, Yb^{3+} or surface-associated organic molecules. The situation can quickly become rather complex with lanthanides whose luminescence involves the $4f^n$ configuration. In these cases, magnetic dipole transitions are allowed and may have intensity of the same order of magnitude as electric dipole transitions. Additionally, some induced dipole transitions are hypersensitive to the environment of the lanthanide ion and apparently follow the selection rules of electric quadrupole transitions, leading them to be referred to as pseudo-quadrupolar transitions.

A 2004 report investigated energy transfer between porous networks of interconnected 18 nm YAG:Ce^{3+} nanocrystals (NCs) and the amine-reactive fluorescent dye tetramethylrhodamine isothiocyanate (TRITC) (Wuister et al., 2004). Glycine was used to coat the NCs, bound to the surface through the carboxylate moieties and providing terminal amines for attachment of TRITC. ET for the conjugate was demonstrated through strong emission of TRITC relative to NCs following selective excitation of the NCs, as well as the appearance of a fast initial decay of the time-resolved PL. The ET was estimated using Förster-Dexter theory, giving a "critical distance" (equivalent to R_0) of 7 nm, resulting in energy transfer rates of up to 10^8 s^{-1} for Ce^{3+} sites within 5 nm of the NC surface, supposed to be ∼90% of the total Ce^{3+} given the NC size.

Electrostatic complexes of $CePO_4$:Tb nanorods and Rhodamine B (RhB), using Ce^{3+}-sensitized Tb^{3+} emission to excite RhB, resulted in ET efficiency η up to 0.85 as determined by ratiometric luminescence analysis (Di et al., 2010). Evidence of ET was taken by the quenching of the NP steady-state luminescence and concomitant increase in RhB emission with increasing amounts of RhB. Time-resolved measurements of the $^5D_4 \rightarrow {}^7F_5$ transition of Tb^{3+} also exhibited quenching but did not quantitatively agree, reporting efficiencies lower than those determined by steady-state quenching ($\eta \sim 0.7$ at the highest quenching condition).

A recent (2013) study investigated electrostatic complexes of $LaPO_4$:Ce nanorods and the fluorescent dye coumarin 440 (C-440) using steady-state and time-resolved PL measurements (Kar et al., 2013). The Stern-Volmer sphere of action static quenching model was applied to the steady-state quenching, and the ET efficiency estimated by the ratio of the Ce^{3+} fluorescence lifetimes, giving $\eta = 0.24$ for an estimated 1:47 nanorod:dye ratio. ET was corroborated by an increase of the fluorescence lifetime of the dye, excited at 280 nm, when complexed with the nanorods.

X-ray-induced singlet oxygen production has been investigated with a handful of Tb^{3+}-activated oxide and fluoride nanoscintillators coupled with grafted or encapsulated photosensitizers. In one study, 11-aminoundecanoic acid-coated $La_{0.8}Tb_{0.2}F_3$ NPs were mixed with the water-soluble photosensitizer meso-tetra(4-carboxyphenyl) porphine (MTCP) (Liu et al., 2008), which resulted in an increase in the quenching rate of the anthracenedipropionic acid (ADPA) singlet oxygen probe compared to PS alone under 250 keV X-ray irradiation at 44 cGy/min. Singlet oxygen production was demonstrated using $(Gd_{0.5}Tb_{0.5})_2O_3$ NPs with PS-encapsulating polysiloxane shells (Seve et al., 2012). The photosensitizer 5-(4-carboxyphenyl)-10,15,20-triphenyl-chlorin (TPC) was first conjugated to APTES before reaction of the TPC-APTES with TEOS for shell formation, resulting in varied amounts of covalently bound TPC embedded within the shell. In this case, increasing concentrations of encapsulated TPC resulted in quenching of the TPC PL (directly excited at 414 nm) as well as singlet oxygen production (directly detected through 1270 nm phosphorescence). This result was attributed to migration of excitation energy between TPC molecules terminated at static quenching sites, with a model developed to support the data.

Recently, energy transfer mechanisms and singlet oxygen production under optical and X-ray irradiation were studied using a similar system consisting of Gd-free Tb_2O_3 NPs with the photosensitizer 5-(4-carboxyphenyl)-10,15,20-triphenyl

porphyrin (TPP) grafted to the polysiloxane shells after rather than during their formation (Bulin et al., 2013). The NPs and NP-PS were used in DEG solution. Upon excitation at 300 nm (primarily resulting in $4f^8 \rightarrow 4f^7 5d^1$ transitions in Tb^{3+}), a concurrent decrease of the Tb^{3+} lifetimes (measured at 545 nm) and appearance of long PL lifetimes of the grafted PS (measured at 650 nm) were taken to be indicative of excited Tb^{3+}-PS nonradiative energy transfer. Interestingly, the polysiloxane layer was implicated in the appearance of a broad emission component from the NPs with a peak ∼425 nm that was also involved in efficient, fast energy transfer to TPP under optical excitation, but did not appear under X-ray excitation. Singlet oxygen yields under 44 kV X-ray excitation (from a tungsten anode, providing a dose rate of 5.4 mGy/s) were evaluated with the chemical probes singlet oxygen sensor green (SOSG) and 3′-p-(aminophenyl) fluorescein (APF). SOSG showed a steady increase in signal with both PS alone and NP-PS, with the NP-PS showing a relative increase for irradiation times >10 min. The APF probe corroborated the formation of singlet oxygen by the NP-PS system, supported by competitive quenching of singlet oxygen by addition of NaN_3.

A small number of nanoscintillator-PS conjugate systems have demonstrated measurable enhancements of X-ray irradiation in cancer cell lines. In one study, commercially available Y_2O_3 NPs were modified with 2-chloroethylphosphonic acid (2-CEP) ligands, which were used to form thioether linkages to fragments of the HIV-1 TAT cell-penetrating/nuclear targeting peptide bound to the PS psoralen (Scaffidi et al., 2011). A small but significant downward trend in the growth of PS-3 prostate cancer cells with 2 Gy of 160 kVp or 320 kVp X-rays was seen as a function of particle dose. Another study reported activity of a terbium-doped gadolinium oxysulfide-Photofrin II mixture against glioblastoma cells irradiated with 120 kVp diagnostic X-rays. Radiation alone produced 20% cell suppression, and radiation plus the NP-PS combination over 90% suppression. Interestingly, the particles alone (without Photofrin) protected the cells against X-irradiation.

A theoretical paper investigated the conditions required for a nanoscintillator-photosensitizer conjugate system to produce therapeutically-relevant results, using physical parameters including nanoparticle uptake into cells, enhancement of radiation dose, scintillation light yields, and energy transfer efficiencies (Morgan et al., 2009). These parameters were used to estimate the overall singlet oxygen yield of a NP-PS system with X-ray irradiation. As singlet oxygen is considered to be the primary effector of PDT, its production was taken to be indicative of the potential of conjugates to damage malignant tissue through PS activation. Overall singlet oxygen production Φ_{1O_2} was determined from the product of the scintillation yield φ_s, characteristic of the material and given in photons per MeV of absorbed radiation, the NP-PS energy transfer efficiency φ_{ET}, and the PS singlet oxygen yield φ_p. For an extremely generous value of $\varphi_s > 10^5$ photons/MeV (derived from the energy output of bulk crystals of hygroscopic LuI_3:Ce^{3+}) and somewhat generous values of $\varphi_{ET} = 0.75$ and $\varphi_p = 0.89$, and using the relative X-ray absorption of the NPs, it was determined that to deliver the "Niedre killing dose" of singlet oxygen (reduction of a cell population to 1/*e* fraction, based on *in vitro* measurements of OCI-AML5 leukemia) (Niedre et al., 2002, 2003), only X-ray energies below ∼200 keV (with peak efficiency ∼50 keV) would be effective for reasonable total radiation doses. These results suggest that it would be difficult to produce a dramatic outcome with PDT effects alone.

It has been established that the efficacy of PDT *in vivo* depends on three primary mechanisms: direct tumor-cell killing; damage to tumor vasculature; and provocation of an immune response (in contrast to the immunosuppressive effects of radiotherapy and chemotherapy (Dolmans et al., 2003). If these observations hold true for nanoscintillator-photosensitizer systems, it is conceivable that the optimal targeting and cell-level distributions of such systems may be different from those that rely solely on radiation dose enhancement by nanoparticles (which are most effective in close proximity to cell nuclei). It would also be reasonable to expect that preserving the amphiphilicity of bioconjugated photosensitizers might be beneficial, as the tendency to associate with lipid membranes is known to be a key factor in the activity of free PS molecules (Kessel et al., 1987; Jori and Reddi, 1993). Whether active targeting to tumors improves nanoparticle accumulation in human cancers and/or treatment outcomes remains debatable. There are certainly circumstances in which passive accumulation is insufficient due to the physical properties of the tumor, but the ideal target for human tumors has not been well established (Kobayashi et al., 2013; Moghimi and Farhangrazi, 2014; Nichols and Bae, 2014).

OTHER ALTERNATIVES: CHEMOTHERAPY-NANOPARTICLE CONSTRUCTS

A large number of nanoparticle conjugates to chemotherapeutic agents have been reported, but few of these have been used for radiosensitization. This is somewhat surprising, since traditional chemotherapeutic agents often act as radiosensitizers, and probably just reflects the emerging state of the field. A few reports have targeted metal nanoparticles to cells or tumors using molecules that play an active role in destroying the target cells. In one study, radioresistant melanoma cells were exposed to Au nanorods conjugated to the RGD peptide (Xu et al., 2012). Exposure to MV X-rays decreased integrin expression and rendered the cells susceptible to radiation-induced apoptosis.

Another study showed that nanoparticle preparations of epithelial growth factor receptor (EGFR) antisense oligonucleotides radiosensitized SCCVII murine squamous carcinoma cells (Xu et al., 2012). However, the nanoparticles themselves were a delivery vehicle only, so no synergy was being sought between the particles and their cargo.

In another approach, doxorubicin conjugated to DNA-coated large Au nanoparticles was loaded into MCF-7 breast cancer cells (Starkewolf et al., 2013). Irradiation with X-rays improved cell inhibition by 33% at 10 Gy relative to Dox alone or Au nanoparticles alone. The authors attributed this observation to release of Dox by the radiation.

SUMMARY AND CONCLUSION

Dense inorganic nanoparticles show considerable promise for dose enhancement of radiation therapy and enabling synergistic

co-treatments. Gold nanoparticles are the most studied, though are not yet in the clinic for radiation therapy. Research efforts are underway to increase the efficiency of nanoparticle-based treatments, including physical and chemical optimization of nanoparticles, improved targeting such that total doses can be reduced, and combining ionizing radiation with other therapeutic modalities. Pre-sensitization of tumors with localized heating resulting from illumination of Au nanostructures with infrared light (photothermal therapy) has shown encouraging results. A number of less expensive alternatives to Au have been produced, but have not been subject to the same level of research activity. Oxides and selenides of Pt and Bi have been shown to provide radiation dose enhancement, while those of Gd and Fe also enable magnetism-based imaging, guidance and hyperthermia.

Nanoscintillators consist of a broad class of nanostructures that emit light ranging from the ultraviolet to the infrared upon excitation by ionizing radiation, with spectra that depend primarily on composition. Energy transfer from excited state nanoscintillators to surface-attached photosensitizer molecules allows such a system to improve upon the issue of tissue transmittance encountered with typical PDT, combined with the dose enhancement provided by the dense nanoparticles. If the emitted light is of an appropriate wavelength to be absorbed by photosensitizer molecules, nanoscintillator-photosensitizer bioconjugates have the potential to improve upon the issue of tissue transmittance with typical PDT. Such systems have only recently been reported, but represent another distinct class for combined therapy that requires only ionizing radiation. As these systems have thus far only been studied *in vitro*, and cover many possible material compositions and drug varieties, it is difficult to reach definitive conclusions about their advantages and disadvantages compared to Au. While the raw materials are less expensive than Au in general, the particles tend to be less than half as dense as Au, and provide lower enhancement factors. While the surface chemistry of Au is well established and reliable, oxide and fluoride nanoscintillators have known colloidal stability issues. Certainly, if XRT and PDT effects are determined to be synergistic, such systems may soon become a viable option for nanotherapeutics.

Despite the substantial progress in nanoparticle-assisted therapies in recent years, nanoscale radiosensitization effects have not yet been studied in great detail. Further understanding of the essential principles and interactions will help establish the legitimacy of new undertakings in the burgeoning field of nanomedicine, where clinical applications are just beginning to emerge.

While a good deal of preclinical data on GNRT is available, there are not yet clinical trials in the U.S. Two types of Au nanoparticles have been FDA approved for cancer trials: Au-tumor necrosis factor conjugates (clinicaltrials.gov, NCT00356980) and Au nanoshells for photothermal therapy (AuroLase, currently recruiting, NCT01679470 and NCT00848042 for lung cancer and head and neck cancer, respectively). Hafnium oxide particles are in clinical trials as radiation enhancers (NCT01433068, currently recruiting; drug name NBTXR3).

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Therapeutic antisense oligonucleotides against cancer: hurdling to the clinic

Pedro M. D. Moreno[1]* and Ana P. Pêgo[1,2,3]

[1] Instituto de Engenharia Biomédica, Nanobiomaterials for Targeted Therapies Group, Porto, Portugal
[2] Faculdade de Engenharia, Universidade do Porto, Porto, Portugal
[3] Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

Edited by:
João Conde, Massachusetts Institute of Technology, USA

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Ramon Eritja, Institut de Química Avançada de Catalunya - Consejo Superior de Investigaciones Científicas, Spain

***Correspondence:**
Pedro M. D. Moreno, Instituto de Engenharia Biomédica, Nanobiomaterials for Targeted Therapies Group, Rua do Campo Alegre, 823, Porto, Portugal
e-mail: pedro.moreno@ineb.up.pt

Under clinical development since the early 90's and with two successfully approved drugs (Fomivirsen and Mipomersen), oligonucleotide-based therapeutics has not yet delivered a clinical drug to the market in the cancer field. Whilst many pre-clinical data has been generated, a lack of understanding still exists on how to efficiently tackle all the different challenges presented for cancer targeting in a clinical setting. Namely, effective drug vectorization, careful choice of target gene or synergistic multi-gene targeting are surely decisive, while caution must be exerted to avoid potential toxic, often misleading off-target-effects. Here a brief overview will be given on the nucleic acid chemistry advances that established oligonucleotide technologies as a promising therapeutic alternative and ongoing cancer related clinical trials. Special attention will be given toward a perspective on the hurdles encountered specifically in the cancer field by this class of therapeutic oligonucleotides and a view on possible avenues for success is presented, with particular focus on the contribution from nanotechnology to the field.

Keywords: antisense, oligonucleotides, cancer, therapeutics, nanomedicine

OPENING THE THERAPEUTIC LANDSCAPE BY EVOLUTION OF NUCLEIC ACIDS CHEMISTRY

Oligonucleotides have been under investigation for over 30 years, whilst achieving only two approved drugs. Those were, Fomivirsen, approved by the FDA in 1998 for the treatment of cytomegalovirus retinitis in patients with AIDS, but discontinued for low demand, and Mipomersen, FDA approved in 2013, targeting ApoB100 for the treatment of homozygous familial hypercholesterolaemia (HoFH), a rare genetic disorder that leads to excessive levels of low-density lipoprotein (LDL) cholesterol. These are both single-stranded antisense oligonucleotide drugs (most commonly known as AONs) that together with siRNA (a double-stranded oligonucleotide) make up, at present, the therapeutic antisense oligonucleotide field. In this paper more emphasis will be put on AONs due to their longer time in development and history of clinical trials.

Progress in this field has been proceeding at a steady but somewhat slow pace, driven mostly by the speed at which the different intra and extracellular obstacles encountered by the oligonucleotide drugs are being tackled. The most important hurdles have been (i) the poor stability against extra- and intracellular degradation (mostly by action of nucleases), (ii) inefficient intracellular delivery to target cells or tissues, (iii) inadequate affinity toward the intended target sequence and (iv) potential off-target/toxicity effects. Finally for most applications (v) immunostimulation has also been a matter of concern.

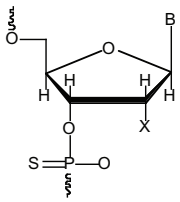
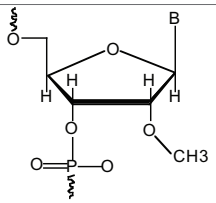
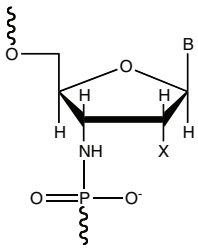
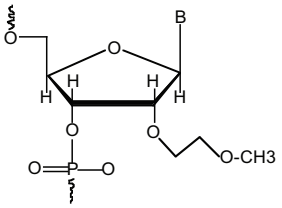
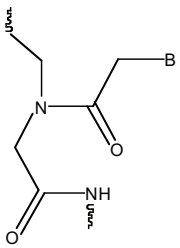
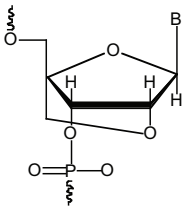
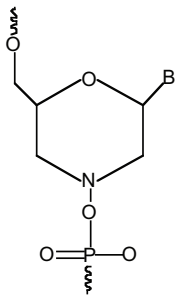
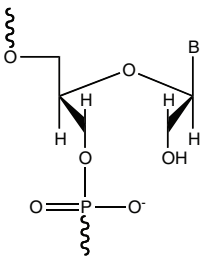
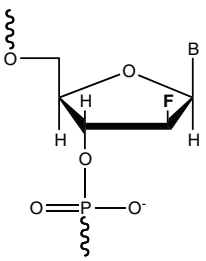
The pursuit of clinically relevant antisense drugs has led the field to develop different types of chemical modifications to native DNA or RNA in an attempt to overcome the aforementioned limitations. Most widely used modifications can be divided in two simple categories: (a) backbone structure and (b) sugar ring modifications (**Table 1**).

The main goals of these chemical modifications have been to achieve increased resistance to degradation by exo- and endonucleases; increase affinity, and in some cases selectivity, toward target RNA/DNA sequences and to modulate the immunostimulation properties of the oligonucleotides.

In the case of phosphorothioate (PS) modification (one of the first and widely used modifications introduced in therapeutic antisense oligonucleotides) (Eckstein, 1967), it has also led to better pharmacokinetics and extended circulation times for AONs systemically applied in a "naked" form (i.e., unprotected by delivery agents). This effect has been attributed to unspecific binding to serum proteins such as albumin (Srinivasan et al., 1995; Crooke et al., 1996; Watanabe et al., 2006).

Mechanistically, AONs work via binding to a specific RNA target sequence resulting in the block of RNA function. This can be achieved through steric hindrance (non-degradative pathway) and concomitant RNA translation block, or target degradation. The latter occurs by the action of an endogenous enzyme, RNase H, or alternatively, by a catalytic cleavage activity embedded into the oligonucleotide itself (e.g., ribozymes and DNAzymes) (Bennett and Swayze, 2010). Notably, the non-degradative mechanism, through steric hindrance, has recently been exploited, with great success, for modulation of pre-mRNA splice patterns by affecting the binding of trans-splicing regulatory factors to the pre-mRNA (Hammond and Wood, 2011; Bestas et al., 2014; Disterer et al., 2014).

Table 1 | Common nucleic acids modifications divided by category.

BACKBONE STRUCTURE	SUGAR RING
 Phosphorothioate (PS)	 2′*O*-Methyl (2′*O*-Me)
 N′3 Phosphoramidate (NP)	 2′*O*-Metoxyethyl (MOE)
 Peptide Nucleic Acid (PNA)	 Locked Nucleic Acid (LNA)
 Morpholino (PMO)	 Unlocked Nucleic Acid (UNA)
	 2′*F*-Arabino Nucleic Acid (2′*F*-ANA)

The mechanism of action of AONs has to be carefully considered when deciding for the type of nucleotide modifications and design of the oligonucleotide (here including number and position of modified nucleotides). Thus, in contrast to PS modifications that maintain the anionic character of oligonucleotides, PNAs and PMOs completely substitute the phosphodiester linkage by either a polyamide backbone (Nielsen et al., 1991) or a phosphorodiamidate group (Summerton and Weller, 1997), respectively, hence being uncharged nucleotide analogs. On the other hand sugar ring modifications can influence the nucleoside conformation promoting the preferential adoption of an A-form (dsRNA type) or B-form (dsDNA type) helix when in a double-stranded structure. In the case of 2′*O*-Me RNA (Kawai et al., 1992; Nishizaki et al., 1997), MOE (Manoharan, 1999) and LNA (Koshkin et al., 1998; Obika et al., 1998), all promote the A-form while 2′*F*-ANA (Berger et al., 1998) the B-form. Contrasting all the aforementioned, UNA, with its unlocked ring configuration, does not impart any conformation restrictions (Pasternak and Wengel, 2011).

All of the abovementioned nucleotide modifications have thus been used with few restrictions when designing steric hindrance AONs, since their incorporation mainly focus on achieving enhanced binding affinity and selectivity toward a target sequence. In contrast, the design of AONs for target degradation through the action of RNAse H has to obey the enzyme's structural preferences for cleaving DNA/RNA duplexes (Minshull and Hunt, 1986; Nakamura et al., 1991). Hence, all modifications too divergent from natural DNA nucleotides need to be carefully considered to not hinder the enzyme action. This can be accomplished by the design of "gapmer" AONs containing the modified nucleotides on the 5′ and 3′ terminus flanking a central unmodified DNA nucleotides stretch (Monia et al., 1993; Stanton et al., 2012). Specifically, in the case of the two approved drugs, Fomivirsen is a PS modified DNA oligonucleotide applied by intraocular injection, whereas Mipomersen is a second generation AON gapmer with MOE modifications at the ends and PS throughout, applied as an intravascular injection.

BRIEF OVERVIEW ON ANTISENSE OLIGONUCLEOTIDES CLINICAL TRIALS RELATED TO CANCER

An increasing number of clinical trials with AONs are ongoing, which shows that the field is rapidly forwarding. In **Table 2** a list of recently completed and on-going clinical trials is presented.

Other studies have unfortunately failed, in different phases, to reach their expected endpoints or to show significant benefit, leading to a stop in the corresponding AON development. Some aspects of antisense technology have contributed to this and are next discussed.

CHALLENGES FOR ANTISENSE TECHNOLOGY—1. UNSPECIFIC MODES OF ACTION

Along their development path, oligonucleotides have unraveled much of their potential but also many of their limitations.

As discussed above, introduction of PS modifications led to the first evidences that antisense drugs could become a reality in a clinical setting, essentially by increasing resistance to degradation and extending circulation times after systemic administration (mostly due to unspecific serum-protein binding). These properties improved the oligonucleotide therapeutic potential, despite some decreased affinity for the target sequence (when comparing to regular DNA oligonucleotides) (Kibler-Herzog et al., 1991). On

Table 2 | On-going and recently completed anti-cancer AON clinical trials.

DRUG	AON (carrier)	TARGET	INDICATION	STATUS	DEVELOPER
Custirsen (OGX-011)	2′-*O*-MOE-PS gapmer ODN ("naked")	Clusterin	(i) castrate resistant prostate cancer; (ii) non-small cell lung cancer	I and (ii) Phase III (recruiting)	OncoGenex
EGFR antisense DNA	Phoshorothioate ODN ("naked")	EGFR	Advanced Head and Neck Squamous Cell Carcinoma	Phase I/II (recruiting)	University of Texas
Apatorsen (OGX-427)	2′-*O*-MOE-PS gapmer ODN ("naked")	Hsp27	prostate cancer; pancreatic; non-squamous non-small cell lung cancer; other	Phase II (recruiting)	OncoGenex
ISIS-STAT3Rx (ISIS 481464/ AZD9150)	cEt-PS gapmer ODN ("naked")	STAT3	Lymphoma; hepatocellular carcinoma	Phase I/II (recruiting)	Astrazeneca (ISIS Pharmaceuticals)
ISIS-ARRx (AZD5312)	cEt-PS gapmer ODN ("naked")	Androgen Receptor	Advanced solid tumors (prostate cancer indications)	Phase I (recruiting)	Astrazeneca (ISIS Pharmaceuticals)
Trabedersen (AP 12009)	Phoshorothioate ODN ("naked")	TGFβ2	(i) Pancreatic Neoplasms, Melanoma, Colorectal Neoplasms; (ii) Glioblastoma; Anaplastic Astrocytoma	(i) Phase I; (ii) Phase IIb (both completed)	Isarna Therapeutics
EZN-2968	LNA-PS gapmer ODN ("naked")	HIF-1α	Advanced solid tumors	Phase I (completed)	Enzon Pharmaceuticals (Santaris Pharma)
LErafAON-ETU	DNA-PS modified at 5′ and 3′ end (lipossome)	c-raf	Advanced Cancer	Phase I (completed)	INSYS Therapeutics Inc

ODN—oligodeoxynucleotide; cEt—constrained Ethyl.

the other hand, this unspecific protein binding feature can potentially lead to associated toxicities or cellular effects not entirely sequence specific, such as complement activation, increased coagulation times and unwanted immune activation (Brown et al., 1994; Krieg and Stein, 1995; Henry et al., 1999; Mou et al., 2001; Krieg et al., 2003; Senn et al., 2005). These effects, however, are most often oligonucleotide length and concentration dependent (Webb et al., 2001). Immune activation, on the other hand, is also enhanced by specific nucleotide sequences (CpG motifs) (Barchet et al., 2008), although this can be minimized by different types of nucleotide modifications (Henry et al., 2000). Nevertheless, immune activation is an important factor that has previously led to erroneous interpretations of data when inhibition of tumor growth was not primarily driven by the antisense mechanism but by the immunostimulatory properties of CpG sequences found in certain AONs (Badros et al., 2005; Gekeler et al., 2006). Regarding potential PS-derived unspecific cellular effects these have been proposed to affect the mechanism of action of an anti-cancer oligonucleotide drug by the down-regulation of several anti-apoptotic proteins and glycolytic enzymes. These were actually seen as important contributors to the apoptotic action (Stessl et al., 2009; Winkler et al., 2010).

Another important concern relates to hybridization dependent toxicity, deriving from exaggerated pharmacological action (a consequence also seen with any other chemical drug), or off-target hybridization. The latter can be minimized by designing the antisense drug taking into account a detailed bioinformatics analysis for identification of both, genes with perfect matches or with partial complementarity (looking out for 1–3 mismatches as the most relevant ones) (Bennett and Swayze, 2010).

The above considerations have raised some difficulties, especially *in vivo*, for the exact prediction of the mechanism of action of an antisense drug and are among the causes probably hampering a more resolute demonstration of the therapeutic relevance of antisense drugs toward not only cancer but also other diseases in general. This concern can be demonstrated by the case of the antisense drug LY2275796 (a second generation AON with PS and MOE modifications targeting eIF-4E) where, besides target gene downregulation, housekeeping genes were considerably affected as well, raising the question to whether the antisense action was sequence specific or also mediated by off-target effects (Hong et al., 2011).

This scenario only reinforces the need for an in-depth pharmacologic and pharmacokinetic analysis at the preclinical stage of AON development.

CHALLENGES FOR ANTISENSE TECHNOLOGY—2. DELIVERY

The efficient and targeted delivery of nucleic acid therapeutics is seen as, if not the biggest, one of the most important challenges for this class of drugs. The most commonly used nucleic acids drugs (namely, plasmid DNA, siRNA and AONs) have specific features influencing their cellular uptake and delivery vector development. AONs, due to the short chain size have very low charge density, in addition, being single-stranded, they have the

aromatic bases exposed (not buried inside a double helix), which confers a slight hydrophobic character to the molecule. These properties, enable some level of interaction with the cell membrane, which can be further potentiated by PS modifications, making possible, although still extremely inefficient, their use without the help of any vector formulations (Watts and Corey, 2012). This has been recently emphasized by the demonstration that short LNA modified AONs were able to sustain gene downregulation in a large variety of cell lines when administered *in vitro* unassisted by transfection agents (also referred as gymnotic delivery), although some cell lines still seem to be completely refractory to this type of AON uptake (Stein et al., 2010). The results obtained by gymnotic delivery seem to correlate well with the obtained *in vivo* gene silencing efficiencies for the "naked" LNA administration; in fact, a better prediction of *in vivo* potency was obtained in comparison to data resulting from transfection-mediated *in vitro* AON delivery, a more standard method to preliminarily analyze AON efficiency (Stein et al., 2010). A similar study showed downregulation of different cancer gene targets, by the gymnotic delivery of LNA-AON in over 30 cell lines, although discrepancies between both studies are seen when relating intracellular localization of the AONs (nuclear vs. cytoplasmatic) and efficient down-regulation activity (Zhang et al., 2011).

Despite several studies demonstrating some activity when using "naked" AONs *in vivo*, and their wide tissue distribution, it has also been realized that these preferentially accumulate in the liver and kidney and to a lesser extent in spleen, lymph nodes and bone marrow (Agrawal et al., 1995; Iversen et al., 1995; Graham et al., 1998; Geary, 2009; Straarup et al., 2010). Liver, as a primary location of oligonucleotide accumulation has received a greater level of attention with some of the most promising AON trials taking advantage of this effect, as seen with Mipomersen (Hovingh et al., 2013). Liver accumulation has been attributed to the role of this organ in clearance by the reticulum endothelium system (RES). This results from the abundant presence of phagocytic Kupffer cells, together with the high blood flow received and, importantly, the existence of a fenestrated vasculature with an average 100–200 nm pore diameter between endothelial lining cells (Wisse et al., 2008). It should be noted that the pharmacokinetics of AONs are dependent on chemistry, with the most favorable properties relating to the presence of PS linkages and the polyanionic character of the molecules. Thus, AONs based on PNA and PMO when administered as "naked" formulations *in vivo*, are rapidly cleared from circulation while showing poorer tissue distribution (Dirin and Winkler, 2013).

Tumor tissue also shares some of the abovementioned features, specially regarding its specific microvasculature characteristics (*viz.* for solid tumors). Fenestrations of 100–700 nm have been found in some tumor vessels, which together with a poor lymphatic drainage give rise to the enhanced permeability and retention effect (EPR) (Jang et al., 2003), responsible for the accumulation of macromolecules or nanoparticles in tumors. Another effect to consider is the usually high interstitial fluid pressure (IFP) in tumors that obviates the normal rapid convective flow from blood to the tissue interstitium (due to osmotic and hydrostatic pressure differences). This effect is counterproductive in terms of drug accessibility to the tumor tissue, which then has to rely in slow diffusion processes. A dense structure of interstitial matrix and cells also mounts a final barrier to the diffusion process (Chauhan et al., 2011). Finally, the uneven leakiness of vessels found in tumors further contributes to a highly heterogeneous process of drug penetration. Another consideration is that the larger the tumor the bigger the regional differences within the tumor itself. This is illustrated by the presence of a necrotic core with an almost complete absence of blood flow, a seminecrotic region with poor blood flow within un-branched vessels, a stable region with branched vessels and good flow and an active angiogenic front where blood flow is variable and can be substantially higher than in surrounding host normal tissues (Jain, 2012).

These hindrances can result in AONs despite reaching tumor tissue, not being able to accumulate to a significant extent in the tumor tissue, with the additional drawback of distributing unevenly throughout the tissue (Plenat et al., 1995; Delong et al., 1997; Devi et al., 2005).

Certainly these delivery issues hamper a more effective translation of anti-cancer antisense oligonucleotides to the clinic.

PERSPECTIVES ON AON VECTORIZATION FOR CANCER THERAPEUTICS

Given the wide tissue distribution properties of AONs and their preferential accumulation in organs other than tumor tissue, this can lead to the necessity of using high amounts of AONs in order to reach a meaningful biological effect, raising concerns due to presence of high AON concentrations in unspecific tissue/organs. In addition, although some level of localization to tumor tissue is attained due to the EPR effect, there can be a large heterogeneity in the targeting and distribution of AONs between tumors and within the same tumor. Not achieving a homogeneous and abundant distribution of AONs to the entire tumor can result in differential intracellular concentrations of the AON affecting functional efficiency and ultimately leading to some cells evading the anti-cancer action.

The development of nanocarriers for AON delivery could have a positive contribution in AON anti-cancer efficiency while minimizing toxicity, although their utility must be evaluated in a case-by-case basis. Nanoparticle systems will be also affected by inter and intra-tumor heterogeneity, where differences between tumor mass strongly influence the EPR and IFP effects. In fact, the EPR effect is more prevalent in tumors of 100 mm^3 which limits its use when targeting small or unvascularized primary or secondary tumor (metastases) (Adiseshaiah et al., 2010). While AONs associated with nanoparticle systems can take greater advantage of the EPR effect, when "naked" administration is employed these will be affected to a wider extent by IFP similarly to small drugs Interestingly, this could mean that free AONs could have an advantage when dealing with a tumor with a less disturbed vascular architecture or when tumor vasculature normalization drugs are used (Juliano et al., 2009; Chauhan et al., 2012). This view can, however, be too simplistic as shown in a work dealing with imaging and modulation of AON microdistribution in solid tumor xenografts (Mocanu et al., 2007). It was seen that a drug-induced decrease in IFP was not accompanied by an expected improved distribution of the AON, in contrast to what has been reported

for some small drugs. This was attributed to a strong association of the AON with regions of necrosis/hypoxia or due to the effect of the drug promoting neovascularization and the less permeable status of the newly formed vessels. Also, one could reason that the tumor matrix and the specific collagen content along with the status of other fibrilliary proteins could affect distribution of AONs (Netti et al., 2000; Mocanu et al., 2007), especially when dealing with PS-AONs due to their unspecific binding properties. In contrast to tumor normalization, the EPR effect can be transiently augmented by modulation of blood pressure and local increase of blood flow through the use of angiotensin-II-induced hypertension and nitric oxide releasing agents (Fang et al., 2011). In this way uptake of nanoparticle systems could be favored.

In terms of available systems for vectorization of AONs these can be divided in nanoparticle systems formed by interactions of different carrier formulations with the AONs or nanoconjugates where AONs are covalently linked to different functional molecules (e.g., peptides, sugars) (Juliano et al., 2012; Yin et al., 2014).

Carrier formulations that have been frequently used for delivery of different nucleic acids comprise cationic lipids and polymers. The basic driving force of complex formation is the electrostatic interaction. In brief, the carrier system needs to (i) protect the nucleic-acid from extracellular and intracellular degradation, until it reaches its target, (ii) achieve a prolonged circulation time in order to be accumulated in the location of interest, (iii) efficiently interact with the cellular membrane to promote uptake (generally through endocytosis processes), (iv) promote escape from endocytic vesicles and finally (v) dissociate from the active nucleic-acid in order for it to function (Yin et al., 2014).

Cationic lipids generally used with nucleic acids (forming lipoplexes) comprise DOTMA, DOSPE, DOTAP, but also neutral lipids such as the fusogenic DOPE have been incorporated to improve transfection efficiency (Simoes et al., 2005). Some of these lipids have been studied specifically with AONs (Jaaskelainen et al., 2000; Meidan et al., 2001; Gokhale et al., 2002) but few have been utilized in pre-clinical or clinical work. A liposome formulation of c-raf antisense oligonucleotide constitutes the first example of an AON-lipoplex taken into clinical development stages (Zhang et al., 2009).

Polymers have been also used. These have an immense chemical diversity and are easy to chemically manipulate thus enabling tuning of properties by functionalization. Some examples of polymeric systems that have been utilized are poly(L-Lysine) (Stewart et al., 1996) and poly(ethylene imine) (Seong et al., 2006). However, some issues regarding efficiency and toxicity have warranted the development of other systems based on natural and biodegradable polymers such as chitosan (Gomes et al., 2014).

Also worth mentioning are delivery systems based on inorganic nanoparticles, an emerging field, of which, gold nanoparticles are perhaps the most representative ones (Ding et al., 2014). A detailed view on the intracellular transport (e.g., understanding its endocytic route) (Wu et al., 2014) and careful evaluation of toxicity profile (e.g., genotoxicity, membrane damage) (Alkilany and Murphy, 2010) can provide important information to the advancement of the technology into clinical development.

Taking into consideration the previously mentioned tumor features, some design specificities should be taken into account when implementing an AON-nanocomplex strategy as anti-cancer therapeutic platform. Regarding size, the smaller the particle the better the intra-tumoral transport (<10 nm), however the EPR effect will be less significant than for bigger particles (10–200 nm) (Netti et al., 2000). On the other hand, particles on the higher size range will present a limited capacity to extravasate from vessel pores, but for the same reason will be more specific. Also, bigger sizes determine a higher clearance by the RES, although this can be counteracted by steric stabilization through poly(ethylene glycol) surface modification (Van Vlerken et al., 2007).

Surface charge also plays a crucial role. While cationic particles tend to target tumor endothelium and exhibit a higher vascular permeability than neutral or anionic ones, the fastest and more homogenously distribution in tumor interstitium is seen for the neutral particles. Presence of charge in particles contributes to aggregation with different components of the tumor matrix thus hindering transport. Accordingly, neutral or zwitterionic particles, or even particles with the property to change charge according to the microenvironment should perhaps be the best options (Chauhan et al., 2011). Shape, an often-overlooked property, likewise affects transport. Here factors such as rigidity and form (spherical vs. rod) come into play with flexible nanometer-sized particles showing, in principle, better transport characteristics (Chauhan et al., 2011).

In conclusion, the field of anti-cancer AONs is rapidly advancing, supported in part by the growing number of chemical modifications that conferred superior properties to AONs. However, specific and efficient delivery to tumors is still of uttermost importance. Uniform distribution throughout the tumor is an important challenge particularly due to intra-tumoral regional specificities and a progressive microenvironment. A further challenge lies in the dynamic nature of tumors that may correlate with temporal and spatial changes in expression of the AON target genes.

Multi-gene targeting AONs and efficient tumor targeting vectorization systems will, thus, be of uttermost importance in the development of a successful anti-cancer AON strategy.

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Cancer immunotherapy: nanodelivery approaches for immune cell targeting and tracking

João Conniot[1], Joana M. Silva[1], Joana G. Fernandes[1], Liana C. Silva[1], Rogério Gaspar[1], Steve Brocchini[2], Helena F. Florindo[1]* and Teresa S. Barata[2]*

[1] *Faculdade de Farmácia, Instituto de Investigação do Medicamento (iMed.ULisboa), Universidade de Lisboa, Lisboa, Portugal*
[2] *EPSRC Centre for Innovative Manufacturing in Emergent Macromolecular Therapies, UCL School of Pharmacy, London, UK*

Edited by:
João Conde, Massachusetts Institute of Technology, USA

Reviewed by:
Min-Ho Kim, Kent State University, USA
Jinjun Shi, Havard Medical School, USA

****Correspondence:***
Helena F. Florindo, Faculdade de Farmácia, Instituto de Investigação do Medicamento (iMed.ULisboa), Universidade de Lisboa, Avenida Professor Gama Pinto, Edificio E, 1649-003 Lisboa, Portugal
e-mail: hflorindo@ff.ul.pt;
Teresa S. Barata, EPSRC Centre for Innovative Manufacturing in Emergent Macromolecular Therapies, UCL School of Pharmacy, 29-39 Brunswick Square, London WC1N 1AX, UK
e-mail: t.barata@ucl.ac.uk

Cancer is one of the most common diseases afflicting people globally. New therapeutic approaches are needed due to the complexity of cancer as a disease. Many current treatments are very toxic and have modest efficacy at best. Increased understanding of tumor biology and immunology has allowed the development of specific immunotherapies with minimal toxicity. It is important to highlight the performance of monoclonal antibodies, immune adjuvants, vaccines and cell-based treatments. Although these approaches have shown varying degrees of clinical efficacy, they illustrate the potential to develop new strategies. Targeted immunotherapy is being explored to overcome the heterogeneity of malignant cells and the immune suppression induced by both the tumor and its microenvironment. Nanodelivery strategies seek to minimize systemic exposure to target therapy to malignant tissue and cells. Intracellular penetration has been examined through the use of functionalized particulates. These nano-particulate associated medicines are being developed for use in imaging, diagnostics and cancer targeting. Although nano-particulates are inherently complex medicines, the ability to confer, at least in principle, different types of functionality allows for the plausible consideration these nanodelivery strategies can be exploited for use as combination medicines. The development of targeted nanodelivery systems in which therapeutic and imaging agents are merged into a single platform is an attractive strategy. Currently, several nanoplatform-based formulations, such as polymeric nanoparticles, micelles, liposomes and dendrimers are in preclinical and clinical stages of development. Herein, nanodelivery strategies presently investigated for cancer immunotherapy, cancer targeting mechanisms and nanocarrier functionalization methods will be described. We also intend to discuss the emerging nano-based approaches suitable to be used as imaging techniques and as cancer treatment options.

Keywords: nanosystems, cancer, targeted delivery, cell tracking, immunotherapy

INTRODUCTION

Cancer is a heterogeneous disease that results from a multi-step process, characterized by uncontrolled tumor cell proliferation, invasion and metastasis. Tumor cells have also the ability to evade cell death (Fernald and Kurokawa, 2013) and to escape immune system surveillance (Zitvogel et al., 2006).

Despite improvements in diagnosis and therapies, cancer is still the most fatal disease worldwide with 11.5 million deaths being predicted in 2030. Strategies for cancer treatment include chemotherapy, radiotherapy, immunotherapy and surgery (Wu et al., 2014). Many of these approaches are unspecific with severe side effects (Peer et al., 2007). More effective and specific alternative treatments continue to be needed. In fact, it has been described that those single treatment regimens have limited chances to eliminate cancer cells in a permanent manner due to its heterogeneous nature (Hanahan and Weinberg, 2000; Helmy et al., 2013).The success of cancer therapy is dependent on the development of additional strategies to overcome severe side effects, drug resistance and circumvent tumor evasion mechanisms (Girardi et al., 2001; Dunn et al., 2002; Koebel et al., 2007; Chen et al., 2014b; Xu et al., 2014).

Although the general body immune response is often not robust enough to escape to cancer cell tactics (Palucka and Banchereau, 2012), our understanding of tumor immunology has been evolving. It is accepted that tumor cells, parts of tumor cells or even specific substances isolated from tumor cells can be recognized by the immune system, which can then respond to these malignant cells. The possibility for immune system-based responses has brought new insights into the development of novel cancer immunotherapy treatments. Immunotherapy has begun to meet its promise for cancer treatment. Monoclonal antibodies (mAbs) to specific targets that are engaged with tumor mechanisms are used clinically, including alemtuzumab (lymphocytic leukemia) and trastuzumab (breast cancer) (Kirkwood et al., 2012). Additionally, cancer vaccination has shown encouraging preclinical results and has also been extensively explored, being

mostly directed to the destruction of tumors by strengthening the immune system (Rosenberg, 2001; Palucka and Banchereau, 2012).

The recognition of the crucial role of T-lymphocytes in cancer for immune-mediated treatments has contributed to the exhaustive characterization of tumor-associated antigens (TAAs). Of particular interest are the cytotoxic T Lymphocytes (CTL), which upon antigen recognition can selectively target and destroy malignant cells presenting epitopes which have been recognized. However, their isolated response is often not enough and the development of an optimal cancer vaccine seems to be dependent on an effective stimulation and cooperation between CTL and T helper (Th) cells specific for a tumor epitope (Fong and Engleman, 2000; Banchereau et al., 2001; Palucka and Banchereau, 2012).

In addition to the evolution of tumor immunology, there has been progress in the development of nanodelivery systems. These systems have the potential to overcome some of the drawbacks of current chemotherapy and radiotherapy therapies. As reviewed by Chow and Ho (2013), nanosystems can display improved pharmacokinetics and targeting of tissues and cells to enhance efficacy, specificity and lower toxicity. Accordingly, nanosystems designed to target immune molecules and cells may allow the development of approaches that will use the patient's immune system as a more specific tool to fight cancer.

Nano-based platforms have also been explored for immune cell labeling, using fluorescence and molecular imaging techniques. As a result, immune cell mechanisms engaged in cancer development and tumor metastasis can thus be better understood, guiding the development of advanced platforms able to specifically target and track immune cells.

CANCER AND THE IMMUNE SYSTEM

INNATE AND ADAPTIVE IMMUNITY TO CANCER

The immune system is composed of two main branches—innate and adaptive immune responses. The innate immunity is a non-specific first line defense of our body against antigens. It comprises anatomic, physiologic, phagocytic, and inflammatory barriers, such as skin or macrophages and neutrophils. On the other hand, adaptive immunity is a highly specific component of the immune system, which is stimulated by a specific antigen challenge to the organism. Still, the latter is not independent from the innate response, since antigen-presenting cells (APCs), involved in innate immunity, play a pivotal role in specific immunity activation (Roitt and Delves, 2001; Kindt et al., 2006).

Dendritic cells (DCs), along with macrophages and B lymphocytes, are described as APCs (Roitt and Delves, 2001; Gogolak et al., 2003; Kindt et al., 2006). DCs are the most powerful "professional" APCs, being present in the majority of mammalian tissues and acting as an interface between innate and adaptive immunity. They control and regulate the immune system. DCs are organized in an intricate network throughout the human lymphatic and non-lymphatic tissues, having different functions, depending on their stage of maturation (Banchereau et al., 2003; Bodey et al., 2004; Palucka and Banchereau, 2012). Non-activated immature DCs capture antigens and induce tolerance in the steady state, whereas mature antigen-loaded DCs can prime an antigen-specific immune response. DCs can also be categorized in three main subsets—(i) Langerhans cells (LCs); (ii) interstitial DCs (intDCs) and (iii) plasmocytoid DCs (pDCs). Though all subsets derive from the same precursor cells—CD34+ hematopoietic stem cells, found in the marrow—they are originated from two major distinct pathways (Banchereau et al., 2003). LCs and intDCs arise from the myeloid pathway, are CD11c+ and both produce IL-2. LCs are present in stratified epithelia, like skin and upper airways, whilst intDCs may be found in all other tissues. Additionally, intDCs can secrete IL-10 and elicit naïve B cell differentiation (Gogolak et al., 2003). The other parallel pathway originates phenotypically CD11c− pDCs with the ability to produce high amounts of type I interferon and to modulate T cell differentiation (Gogolak et al., 2003).

In tumor immunology, DCs are crucial for the presentation of TAAs and to stimulate the immune system after DC activation (Palucka and Banchereau, 2012). DCs patrol the different tissues, processing exogenous and endogenous antigens that are then presented to T lymphocytes, after DC maturation. The maturation process of DCs can be induced directly through "danger signals" detected by pattern recognition receptors (PRRs) or triggered by the presence of inflammatory mediators, such as TNF-α or IL-1β (Bodey et al., 2004).

Antigen presentation to T lymphocytes by DCs occurs through T-cell receptors (TCRs) that recognize antigens bound to major histocompatibility complex (MHC) molecules. MHC proteins can be sorted in two main groups: MHC class I—expressed on the membrane of the majority of nucleated cells in vertebrates—and MHC class II, only found in APCs (Levine and Chain, 1991; Bodey et al., 2004). After the contact of a naïve T cell with MHC-antigen complex, T cells proliferate and differentiate in both memory T cells and effector T cells. Effector T cells may be divided in T helper (Th—CD4+) or T cytotoxic (Tc—CD8+) cells (Guermonprez et al., 2002; Gogolak et al., 2003). The stimulation of Tc cells can lead to the generation of CTLs that secrete low levels of cytokines, unlike Tc cells. However, they display cell-killing action, controlling and eliminating cells that exhibit any type of antigen, such as infected cells or tumor cells (Gogolak et al., 2003). Other innate lymphocytes subsets, such as γδ T cells, natural killer (NK) and natural killer T (NKT) cells, have been reported as being engaged in a complex immunomodulatory network, displaying anti-tumor activity. Preclinical studies described that NKT cells can exhibit anti-tumor or immune-regulatory mechanisms (Gajewski et al., 2013).

The interaction among B cells, T cells and mature DCs results in an integrated immune response. Therefore, DC migration from the tumor site of antigen capture to secondary lymphoid organs can thus greatly broaden antigen-specific T cell responses, promoting effective anti-tumor immune responses that will lead to tumor rejection and regression (Palucka and Banchereau, 2012).

A promising nano-based strategy has been designed in order to develop synthetic DCs for T cell activation and immunotherapy, based on semi-flexible and filamentous polymers (Mandal et al., 2013). Effective antitumor-immune responses are thus dependent on the development of alternative systems to deliver antigens to DCs and promote their presentation to T cells. These factors are

important to bear in mind when developing an effective vaccine (Gajewski et al., 2013).

CANCER IMMUNE REGULATION AND EVASION MECHANISMS

Cancer immunosurveillance

Paul Ehrlich proposed the concept of the immune system as a useful strategy against cancer, in the beginning of the Twentieth century (Ehrlich, 1909). Some decades after, Thomas and Burnet postulated the immunosurveillance theory based on Ehrlich's hypothesis. Cancer arousal was suggested to be caused by the lack of efficiency of the immune system or the modification in antigen expression of tumor cells, leading to its evasion (Burnet, 1957; Thomas, 1982). Thomas and Burnet also claimed that anti-tumor immune response generally happens at an early stage of the cancer development (Burnet, 1957; Thomas, 1982). Therefore, once the tumor has grown, it escaped the immunosurveillance barrier and started developing additional mechanisms to evade from the immune system (Ahmad et al., 2004). Nevertheless, Strutman's later studies showed that cancer susceptibility of immune-competent mice was similar to that observed in mice with major immunodeficiency, setting against the immunosurveillance hypothesis (Shankaran et al., 2001; Dunn et al., 2002). In the beginning of this century, the immunosurveillance hypothesis was revised, as several studies have shown that the immune system may not only destroy tumor cells but also shape their phenotypes, leading to reduction of immunogenicity (Shankaran et al., 2001; Dunn et al., 2002; Schreiber et al., 2011).

Currently, there is increasing evidence that tumor cells can be recognized and destroyed by the immune system, as developing tumor cells often co-express tumor antigens and ligands for activating receptors (Schreiber et al., 2011). Therefore, it is important to describe which immune components display major roles in tumor rejection. It is also important to clarify the appropriate time and efficient type of action (Swann and Smyth, 2007).

Cancer immunoediting and cancer-related inflammation

As reviewed by Schreiber et al. (2011), cancer immunoediting can be divided in three different phases: "elimination," "equilibrium" and "escape."

In the first stage—"elimination"—both innate and adaptive immunities act combined to identify the formation of tumor cells and to destroy them, resembling the immunosurveillance theory. Although many mechanisms are still poorly known, it has been reported that cytokines, "danger signals" and DCs have important roles in this phase (Sims et al., 2010; Vesely et al., 2011). It has also been suggested that the required components for an effective "elimination" depend on specific characteristics of the tumor cells, such as its origin or anatomical location (Sims et al., 2010). If the "elimination" stage is well succeeded, tumor cells are destroyed, constituting an endpoint for cancer immunoediting (Schreiber et al., 2011; Vesely et al., 2011).

The next stage—"equilibrium"—is described as a period of tumor latency. In other words, when a tumor cell survives the elimination phase, the adaptive immune response can control tumor cell growth and shape its immunogenicity. "Equilibrium" is believed to be the longest phase of cancer immunoediting process. It seems to allow cancer cells to reside in patients' body even decades before it restarts to grow and become clinically evident (Schreiber et al., 2011; Vesely et al., 2011).

The third phase—"escape"—occurs when tumor cells have developed the ability to evade the mechanisms of recognition of the immune system and/or their elimination. Tumor cells are thought to progress from "equilibrium" phase to "escape" through several mechanisms and/or pathways. For instance, an alteration in immune system response, which may be triggered by cancer-induced immunosuppression or a change in tumor cells induced by immunoediting, or even immune system deterioration (Schreiber et al., 2011; Vesely et al., 2011).

Cancer immune evasion mechanisms

Cellular immunity has been shown to play a major role in the control of tumor generation. Even though, recent findings revealed that tumors often manage to evade it through several different mechanisms. It has been reported that there is a reduction or even loss of MHCI molecules, mostly associated to gene mutations or impairment of MHCI-dependent antigen processing (Garrido and Algarra, 2001; Ahmad et al., 2004; Vesely et al., 2011). In addition, an antigenic drift in cancer cells has lately been observed and appears to be related with the mutation, loss or down-expression of TAAs in tumor cells (Uyttenhove et al., 1997; Ahmad et al., 2004). Similarly, the lack or reduction of the expression of co-stimulatory patterns by tumor cells direct T lymphocytes to an anergy state. These mechanisms altogether seem to reduce and difficult the detection of cancer cells by CTLs and NK cells, which consequently leads to tumor growth (Ahmad et al., 2004).

Alterations in apoptotic receptor signaling seem to help tumor cells to evade the immune system. Molecules such as phosphatidylinositol 3-kinase (PI3K), protein kinase B and Fas ligand (FasL) have modified expression and might be implicated in this process (Davidson et al., 1998; Osaki et al., 2004).

Tumor eradication is also dependent on the manipulation of immunosuppressive properties of tumor microenvironment, where inducing and suppressing cytokine imbalance impairs DC activation and maturation, compromising immune cell effector properties and supporting tumor growth. Tumor cells can indeed secrete immunosuppressive molecules, including vascular endothelial growth factor (VEGF), IL-10 and transforming growth factor-β (TGF-β) (Fortis et al., 1996; Tsushima et al., 1996; Oyama et al., 1998). VEGF appears to be responsible for down-regulation of NF-κB expression, which interferes in DC maturation and differentiation, limiting the immune response against tumor cells (Oyama et al., 1998). On the other hand, TGF-β1 is an immune suppressive cytokine involved in the conversion of CD4+T cells into immunosuppressive T regulatory (Treg) cells that are mainly produced by DCs and tumor cells (Zou, 2005).

These immunosuppressive molecules are interesting targets to achieve tumor growth inhibition and might be a very useful tool for cancer immunotherapy. The use of nanoparticles (NPs) containing small interfering RNA (siRNA) to knock-down TGF-β in the tumor microenvironment has resulted in increased levels of CD8+ T cells and lower number of Treg cells, leading to tumor growth inhibition by 52% (Xu et al., 2014). A similar strategy using polyethylenimine-capped silica NPs carrying VEGF siRNA has been designed as a highly effective approach for lung cancer

growth suppression and metastasis (Chen et al., 2014b). High levels of indoleamine 2,3-dioxygenase have also been found in tumor microenvironment, reducing tryptophan pool levels, which drive T lymphocytes to be arrested at G1 phase of the cell cycle (Ahmad et al., 2004).

TUMOR MICROENVIRONMENT: TUMOR-INFLITRATING IMMUNE CELLS AND RELATED REGULATORY PATHWAYS

The progress of cancer disease results from several mechanisms developed by tumors to evade antitumor immune responses (Section Cancer immune evasion mechanisms), which has been associated mostly to tumor microenvironment molecular pathways and infiltrating cells at this particular region, rather than the ignorance and defects of anti-tumor T cells (Gajewski et al., 2013; Ma et al., 2013). In fact, the presence of different cells and their dynamic interaction with malignant cells have a profound effect on tumor progression (Mishra et al., 2010; Bussolati et al., 2011; Cortez-Retamozo et al., 2012; Rahir and Moser, 2012).

It is widely accepted that the density of T cell infiltrates within tumor microenvironment is the most important factor to predict cancer patients' survival (Eerola et al., 2000; Oble et al., 2009; Mahmoud et al., 2011). Nevertheless, macrophages are also currently recognized as a fundamental cell type. As a heterogeneous population, its dual function toward cancer is determined by their polarization status (Mantovani and Sica, 2010). Macrophages are regulated by transcription factors, which will lead to different phenotypes of tumor-associated macrophages (TAMs). M1 and M2 have been already characterized, being associated to the pathogenesis of several diseases, namely inflammatory and tumor diseases (Sica et al., 2006). Indeed, pro-inflammatory M1 macrophages, after being activated by IFN-γ, favor Th1 immune cell activity and potentiate the eradication of malignant cells. On the other hand, M2 phenotype enables Th2 immune responses and regulate tissue repair, presenting pro-tumoral abilities in several tumor types (Sica and Mantovani, 2012; Cornelissen et al., 2014). Moreover, the production of several cytokines, such as IL-1, IL-6, IL-10, VEGF, and TGF-β by M2 TAMs elicits the proliferation and metastasis of tumor cells (Biswas and Mantovani, 2010). As a result, it has been described that the number of M2 macrophages and the overall M2/M1 ratio of TAMs are important predictors of survival for distinct types of cancers, namely melanoma (Erdag et al., 2012; Herwig et al., 2013), ovarian cancer (Lan et al., 2013; Colvin, 2014), T-cell (Niino et al., 2010), and B-cell lymphomas (Nam et al., 2014), breast (Leek et al., 1996) and pancreatic cancer (Ino et al., 2013).

It is important to mention however that the M1 and M2 classification of TAMs is not static, being usually very complex and seems to be dictated by several mediators resultant from cellular cross-talk and environmental conditions (Cai et al., 2012; Escribese et al., 2012; Shime et al., 2012). Even tough, the causes underlying the differentiation of TAMs to M1 or M2 phenotypes are not yet fully understood. Type I interferon pathway seems to be fundamental for the activation of innate immune response against tumor cells. However, the production of type I interferon by DCs remain an underexplored issue (Fuertes et al., 2011).

DCs are also present within tumor microenvironment, where they can recognize and capture live and dying tumor cells (Dhodapkar et al., 2008; Ma et al., 2013). Their presence in tumors of different stages and grades correlates to prolonged disease survival and lower invasiveness, as reviewed in Palucka and Banchereau (2012). Even though, some of this heterogeneous hematopoietic lineage displays anti-tumor effects while others present immunosuppressive functions at tumor site. Actually, tumor-infiltrating DCs functionality may vary according to the combination of environmental factors and pathways within variable tumor site. Among DC subsets, it should be emphasized the role of tumor-infiltrating plasmacytoid DCs (pDCs) and CD8α+ DCs lineage, being the first often related to T cell tolerance, while the latter is in fact particularly efficient in the cross-presentation of antigens via MHCI pathways and thus in cytotoxic T-cell immunity (Hildner et al., 2008; Fuertes et al., 2011; Watkins et al., 2011).

The characterization of different solid tumors, as melanoma, showed the presence of tumor-infiltrating lymphoid cell lineage, including CD8+ T cells. Their function is mainly compromised by immune system-inhibitory pathways at tumor microenvironment, enabling T cell anergy (Gajewski et al., 2013). It has been reported the presence of high amounts of CD4+ Foxp3+ regulatory T cells (Treg cells) that are attracted by the chemokine CCL22 via CCR4 (Toulza et al., 2010; Spranger et al., 2013). However, the function of T-cell subsets within tumor microenvironment is highly complex, depending on several factors, such as the type of receptors primed.

Another hypothesis for the presence of T cells within the tumor microenvironment of certain tumors may be related to the formation of a lymph node-like structure called tertiary lymphoid tissues, where it is possible to find B cells, T cells and activated DCs (Messina et al., 2012). Still, it is not clear if the formation of those lymphoid structures is involved in tumor growth *in vivo*. On the other hand, tumor-infiltrated T cells can express CCL21. CCL21 is related to tumor tolerance by stimulating naïve T cells to which the presentation of TAAs will not be efficient due to the absence of co-stimulatory factors (Shields et al., 2010).

NK, NKT and γδT cells also seem to have an important role in the immunomodulation of tumor microenvironment (Peng et al., 2007; Mishra et al., 2010; Marcu-Malina et al., 2011; Liu et al., 2012). The antitumor effect of NK has been linked to solid and hematopoietic tumors, while γδT cells and NKT cells have been involved in tumor inhibition. However, they show immunoregulatory functions in certain circumstances that are not completely known (Peng et al., 2007; Mishra et al., 2010; Marcu-Malina et al., 2011; Liu et al., 2012). A promising strategy has been focused in the stimulation of DCs by α-galactosylceramide to prime NKT, promoting the production of IFN-γ (Shimizu et al., 2013).

Besides these cells, tumor stroma has also been associated with tumor growth and includes different elements as collagen, endothelial cells, fibroblast and several macrophage subsets, which contributes for tumor immune evasion. In addition, higher levels of angiogenic factors were found in tumors where the presence of tumor-infiltrating T cells is poor (Danhier et al., 2010). The major immunosuppressive mechanisms include the secretion of IL-10, TGF-β, and CCL22 by M2 macrophages (Condeelis and Pollard, 2006). The trafficking of T cells within tumor microenvironment has been related to the secretion of different chemokines

by stromal cells, namely CXCL9 and CXCL10 (Gooden et al., 2011).

Nevertheless, the inherent complexity of the immune regulation within tumor microenvironment and the incomplete definition of those multiple mechanisms demand additional efforts to characterize these processes. Such characterization would support the development of translational alternative immunotherapies (Mellman et al., 2011). For example, the presence of tumor-infiltrating T cells within tumor site may indicate that this particular type of tumor is a potential candidate for an immunotherapeutic strategy due to their ability to support the migration of T cells toward this particular region. However, the multiple factors involved in the immune system inhibition indicate that the use of complementary targeted strategies to improve the presence of anti-tumor T cells and the knock-down of immune inhibitory pathways may lead to optimal therapeutic approaches.

Combinatory approaches for cancer therapy need indeed to consider the successful modulation of the tumor-associated cytokine network and cell communication within tumor microenvironment. This will prevent the inhibition of anti-tumor responses and down-regulate the proliferation of malignant cells. The characterization of these immunoregulatory processes and the deeper understanding of the immunological features within tumor microenvironment have fostered the recognition of biomarkers. Such recognition has been driving the design of novel targeted therapies to block those pathways, including targeted nanomedicines to tumor microenvironment to better avoid off-targeted effects. The anti-CTL4 monoclonal antibody ipilumimab approved in 2011 by the US Food and Drug Administration (FDA) to treat patients with advanced melanoma, constitutes the first successful approach that targets one of those inhibitory pathways (Mellman et al., 2011).

The design of these tumor-targeted systems is also influenced by a variety of specific features presented by this region, when compared to healthy tissues. Among those different properties, vasculature and pH have been the most explored toward the development of alternative and specific therapeutic nanosystems (Fernald and Kurokawa, 2013; Torchilin, 2011). Angiogenesis guarantees the supply of oxygen and different nutrients to tumor cells. It results from the action of different factors, as pro-angiogenic proteins, extracellular matrix proteins and matrix metalloproteinases. This process is fundamental for the progression of the disease and has guided the development of different targeted nanocarriers due to the particular morphology of the blood vessels, as reviewed by Torchilin (2011). In fact, abnormal architecture of blood vessel caused by incomplete angiogenesis allows the retention of different nanodelivery systems specifically at this particular tumor region, due to the so-called "Enhanced Permeability and Retention" (EPR), which will be described in Section Passive Targeting.

CANCER IMMUNOTHERAPEUTIC INTERVENTIONS

Cancer immunotherapy has been explored for some decades. This term is often used to describe treatments based on modulation of the immune system through "active" or "passive" approaches. The concept of immunotherapy relies on specific immune mechanisms and targets, which could confer greater efficacy and specificity with less toxicity. Therefore, improving the presence of anti-tumor T cells and the knock-down of immune inhibitory pathways, leading to optimal therapeutic approaches.

ACTIVE CANCER IMMUNOTHERAPY

Active cancer immunotherapy or cancer vaccination consists in direct stimulation of the patient's immune system so it can act against tumor cells. Unlike infectious disease vaccination, which efficiency is based mainly on neutralizing antibodies and B-lymphocyte response, cancer vaccination depends on the induction of CTL responses and on the administration of TAAs to stimulate a systemic immune response.

Cancer vaccines are expected to induce a tumor specific immune response able to either eliminate the malignant cells or keep it under constant restraint, delaying tumor recurrence and prolonging survival. Both prophylactic and therapeutic vaccine-based cancer therapies have been proposed to enhance a specific immune response to tumor cells, concerning DC activity, as summarized in Vacchelli et al. (2012). It has also been reported the prominence of DCs on CTL induction, thus becoming a striking target for cancer vaccination (Section Strategies for DC Targeting).

The extensive research has led to engineered biotech molecules, such as proteins, peptides, antibodies and oligonucleotides, designed to enhance immune-based mechanisms, being promising players to re-shape the future of immunotherapeutic outcomes. However, as these candidates move toward clinical investigation, it becomes clear that their biological effect depends on the development of a tool able to attain their transport across biological barriers. Accordingly, the potential of these bioactive molecules has pointed nanomedicines as an approach to ensure the target selectivity and safety required for their therapeutic *in situ* efficacy, enabling their clinical application.

As discussed by Silva et al. (2013), an ideal vaccination strategy involves the administration of the most immunogenic TAAs along with the most effective adjuvants, including delivery platforms. This will prime the tumor- specific T cells, induce tumor-specific antibodies and kill tumor cells by host immune effector mechanisms.

Several TAAs have been identified and characterized permitting their use in the design of targeted delivery systems (Bos et al., 2012; Engels et al., 2013). TAAs can be sorted as shared tumor antigens—when present in many types of tumors and with a distinct or absent expression on normal tissues (i.e., MAGE, GAGE and NY-ESO1)- or unique tumor antigens. These antigens result from point mutations or splicing alterations and are expressed only by a specific tumor (Higgins et al., 2009; Pejawar-Gaddy et al., 2010). However, those newly identified antigens, as recombinant proteins, are usually weakly immunogenic, requiring multiple administrations and their association with adjuvants. It has been described that both antigen and adjuvant must act in a concerted way on the same APC, which can be provided by a singular delivery system (Schlosser et al., 2008; Krishnamachari et al., 2011; Raaijmakers et al., 2013).

As previously mentioned, the focus of cancer vaccines is the stimulation of a cell-mediated immunity, rather than humoral

responses. As many TAAs are intracellular proteins, fragments of these peptides must be presented on the cell surface bound to MHC class I molecules to be recognized by the immune system (Henderson et al., 2005). Indeed, after the recognition of TAA-MHCI complexes, in lymph nodes (Manolova et al., 2008), CD8+ T lymphocytes can proliferate and differentiate into CTLs. CTLs are then able to migrate to peripheral tissues to develop contact-mediated cytotoxicity activity and secrete effector cytokines as IFN-γ and TNF-α, leading to local inflammation (Ahlers and Belyakov, 2010).

Pattern recognition receptors, mainly the toll-like receptor (TLR) family, are suitable targets to potentiate the presentation of TAAs through MHCI pathway to CD8+ T cells and increase cancer immunotherapy efficacy. Among TLR agonists, both cytosine phosphorothioate-guanine motifs (CpG; TLR9-ligand), double stranded RNA mimic polyinosinic:polycytidylic acid (poly(I:C); TLR3-ligand) and monophosphoryl lipid A (MPL) have been associated to stronger anti-tumor immune responses (Banchereau et al., 2003; Hildner et al., 2008; Radford and Caminschi, 2013).

Generally, TAAs and TLR ligands carried by polymeric particles have the ability to escape the degradation in endosomes and reach the cytosol in higher concentrations than those administered in soluble form. Those antigens can thus be presented by MHC-I molecules more effectively and for longer periods of time, leading to an effective cellular response, which is fundamental for a successful eradication of cancer cells.

PASSIVE CANCER IMMUNOTHERAPY

Passive immunotherapy is based on the administration of *ex vivo* generated immune effector molecules or cells, such as antibodies and CTLs, respectively. These molecules or cells can target specific receptors, leading to enhanced efficacy of the treatment and also to fewer side effects.

Monoclonal antibodies (mAbs)

Monoclonal antibodies are the main cancer immunotherapy used currently in clinic to treat solid tumors and lymphomas (Krishnamachari et al., 2011). For example, trastuzumab has been used to treat $HER2^+$ breast cancer and adenocarcinoma, whilst alemtuzumab has been applied in chronic lymphocytic leukemia treatment (Lee et al., 2013).

The mechanism of action of mAbs is related to their ability to interfere with both growth factor ligands and receptors or pro-apoptotic targets, inducing apoptosis of cancer cells. Besides, mAbs may activate components of the immune system through Fc-region-based mechanisms. This leads to antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) responses by macrophages and NK cells (Krishnamachari et al., 2011).

The use of mAbs in clinic has been increasing in the last decades. The first generation of mAbs used in cancer therapy was originated from mouse. Their origin often resulted in limited half-life, decreasing mAbs efficacy. Further progresses conducted to the development of chimeric mAbs, with enhanced properties, and then humanized mAbs. Nowadays, fully human mAbs are already available (Lee et al., 2013). Several novel mAbs for different cancer types are presently in clinical trials, as reviewed by Lee et al. (2013). For example, ganitumab—for pancreatic cancer –and necitumumab—for non-small cell lung cancer—are now in phase III of clinical trials.

Adoptive T-cell therapy

This approach is based on the transfer of mature tumor-reactive T lymphocytes to act against tumor cells. Unlike cancer vaccines, this strategy is independent from an immune response elicited by an exogenous antigen. Instead, it relies on the delivery of a great amount of *ex vivo*-expanded cells (Gajewski, 2012; Kirkwood et al., 2012; Helmy et al., 2013).

Adoptive T-cell therapy with tumor-infiltrating lymphocytes (TILs) has been proposed. In a successful study, autologous TILs—T cells with potent antitumor activity found within tumors—were harvested, activated *ex vivo* and reinfused in patients. The total remission was reported in more than 20% of the treated patients (Rosenberg et al., 2011).

Complementary research has been made to improve T-cell adoptive therapies. Genetically engineered T cells are under study, in order to manipulate the properties of the administered T-cell population, such as proliferation and migration characteristics (Liu and Rosenberg, 2001; Hinrichs et al., 2011). Also, T cells have been genetically modified to have antitumor specificity by introducing a T-cell receptor for a particular tumor, as previously described in a review by Helmy et al. (2013).

DELIVERY STRATEGIES FOR IMMUNE CELL TARGETING AND TRACKING

STRATEGIES FOR DC TARGETING

Since the role of DCs in inducing CTL immunity is well established, several studies have been made in order to use DC-based cancer vaccines in tumor immunotherapy.

Ex vivo

These vaccines use isolated CD14+ monocytes or CD34+ DC precursors from an individual. After being isolated, these cells are then cultured and differentiated in immature DCs (Romani et al., 1994; Chapuis et al., 1997). The following process is TAA-loading of DCs, which consists in adding proteins, peptides or tumor lysates to its culture medium or through transfection. Additional maturation stimuli, such as CD40L or pro-inflammatory cytokines, may be used to ensure DCs will be able to induce a strong cellular immune response. Finally, loaded mature DCs are administered back into the patient by intravenous (i.v.), subcutaneous (s.c.), intradermal (i.d.), intratumoral (i.t.) or intralymphatic (i.l.) route (Hamdy et al., 2011).

The use of a tumor cell to stimulate DCs seems to induce a better immune response, but it is limited by a possible induction of autoimmune diseases, due to the lack of antigen specificity among the undefined antigen found at cancer cell surface.

Whichever the type of antigen used to pulse DCs, although it has been reported that this approach is safe and able to induce CTL immunity, the clinical observed goal is low, possibly due to the *in vivo* general complex interactions between immune cells (Rosenberg et al., 2004).

DC therapy involves the isolation, culture and stimulation of patient's monocytes and macrophages *ex vivo* using TAAs (Cho et al., 2011). When administered back to the patient, antigen-loaded DCs will bypass the *in vivo* uptake of tumor antigens. DCs are already activated and therefore they are able to migrate to the secondary lymph nodes wherein they will trigger T cells. However, the relative short half-life of TAA-MHC complexes on DC membrane surface, and the low percentage (3–5%) of DCs that can migrate to the lymph nodes and contact with T cells can contribute to the low rate of success of these vaccines (De Vries et al., 2003; Hamdy et al., 2011). Also, being produced specifically for a particular patient, *ex vivo* DC-based vaccines are a highly complex, laborious, time-consuming and expensive approach. Futhermore, the vaccine quality might depend on the clinic where it is produced, once there are several variable parameters in the process, such as dose of DCs and posology (Hamdy et al., 2011). The type of DCs stimulated, antigen loading method and DC maturation level are also important aspects to be characterized to better understand the adjuvant role of DCs.

In vivo

To overcome the lack of clinical efficacy of *ex vivo* DC-based cancer vaccines, it is extremely recommended to develop an alternative way to target antigens directly to DCs *in vivo*, which can be achieved using peptide-based vaccines. These are mainly based on MHCI peptides, which are simple to produce and administer, and guarantee DC activation and expansion for prolonged periods of time (**Figure 1**) (Cheong et al., 2010; Silva et al., 2013). However, the cytoplasmic delivery of the antigen is limited by low membrane permeability and frequent destruction after intracellular entry, being their immunogenicity considerably lower than the traditional vaccines. Hence, their association to potent adjuvants,

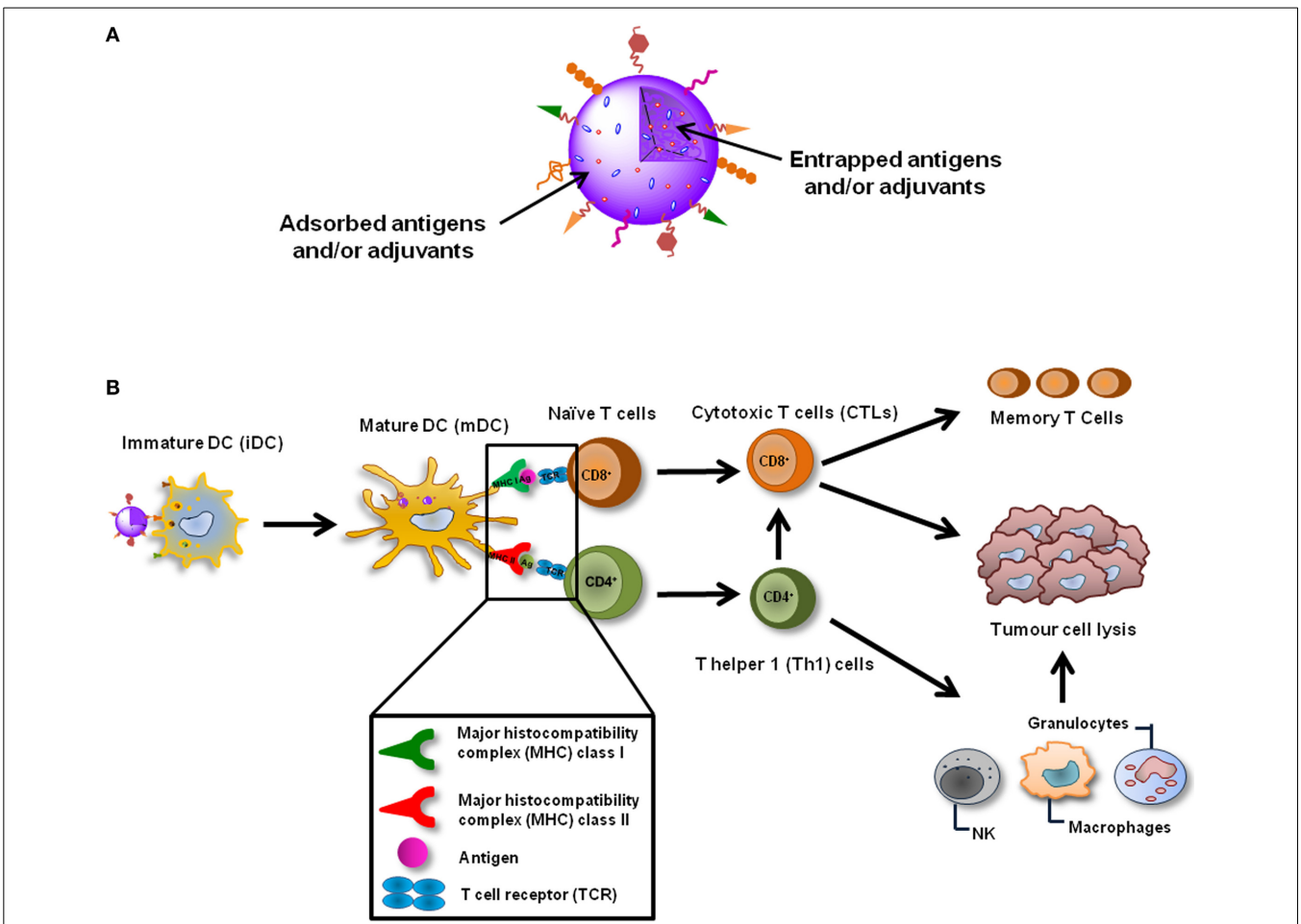

FIGURE 1 | Nanoparticulate cancer vaccines. (A) NPs are able to deliver several TAAs and adjuvants simultaneously, enabling a coordinated activation of DCs. NPs can also be functionalized in order to actively target DCs *in vivo*, increase their cellular internalization and immunogenicity or even target specific intracellular compartments. **(B)** NP-based cancer vaccines can be targeted to DCs *in vivo* and after their internalization induce the maturation of these cells. TAAs and adjuvants are simultaneously released inside the same DC which guaranties its coordinated activation. TAAs are presented trough MHC class I and class II molecules to CD8+ and CD4+ naïve T cells which recognize the processed antigens through TCRs. Activated CD8+ T cells differentiate into CTLs, which can destroy tumor cells, and memory T cells, that are important to avoid recidivism and metastasis. CD4+ T cells should differentiate in Th1 cells, which will potentiate the action of CTLs and will also activate cells of the innate immune system, such as NK cells, granulocytes and macrophages that play a role in the tumor destruction process as well.

as particulate vaccine delivery systems, or immunomodulatory molecules is being widely investigated (Al-Hanbali et al., 2006; Hillaireau and Couvreur, 2009; Sharp et al., 2009; Shahar et al., 2010; Smith et al., 2012).

Numerous studies have demonstrated that these delivery platforms could increase the uptake of antigens and adjuvants by DCs, leading to better immune responses (Diwan et al., 2002; Schlosser et al., 2008; Florindo et al., 2009a). *In vivo* DC-targeted vaccines are able to deliver, within the same platform, both antigens and additional stimuli (i.e., adjuvants) to the same cell in its natural environment, enhancing and maximizing the outcome (Kazzaz et al., 2006). Particulate delivery systems range from micro and nanoparticles, liposomes, to virus-like particles (VLPs). Unlike *ex vivo* DC vaccines, the clinical intervention is limited to vaccine administration, sparing time in fastidious cycles of blood withdrawal and *in vitro* cell culture. Also, it offers on-shelf products, which can be produced at large scale with cost reduction and increased quality.

NANOTECHNOLOGY-BASED APPROACHES AS IMMUNE CELL TARGETED DELIVERY SYSTEMS

Nano-based systems have been described as platforms for targeting and delivery of not only therapeutic agents, but also nanodevices and analytical systems for theranostics. The range of applications of nanosystems can include drug delivery, cancer and gene therapy, as well as imaging and cell tracking through biomarkers and biosensors (Rawat et al., 2006) (Supplementary Material). Nanosystems have been used to increase the resolution of clinical imaging, with improved sensitivity and specificity, leading to earlier diagnostics and real-time results. This may allow the use of prophylactic measures, to avoid the progress of the disease or to greater efficacy of therapies, due to an earlier treatment (Riehemann et al., 2009).

The development of nano-based systems has provided protection strategies for incorporated agents, such as biomolecules—nucleic acids, peptides and proteins—which are generally quickly degraded when administered *in vivo*. Therapeutic agents can be embedded, encapsulated, or even adsorbed or conjugated onto the nanosystems, which can be modified and associated to other adjuvants to achieve an optimized release profile (Mahapatro and Singh, 2011). Usual concerns about the administration of these biomolecules have been eased, since lower doses are generally used and a more restricted distribution is achieved (Rawat et al., 2006). In fact, the widely recognized versatility of nanotechnology strategies allows the accurate design of multifunctional nanocarriers. These, in turn, can be functionalized by ligands of different natures to promote a targeted delivery of their cargo both at cellular and subcellular level.

Nanocarriers can also potentiate the cytosolic delivery of biomolecules as siRNA and miRNA, important gene expression regulators, providing their escape from endo-lysosomal compartments. miRNAs are short oligonucleotides (18–22 nucleotides) and are involved in multiple pathways related to the development and differentiation of cells, and in the pathogenesis of cancer, constituting a valuable target Chen et al., 2014b; Gajos-Michniewicz et al., 2014). However, its *in vivo* application demands the development of cell-specific delivery approaches to promote their biological effect, which are currently underexplored.

The modulation and regulation of the pathophysiology dynamics at the molecular level has enabled nanomedicines to achieve a disease control with an unprecedented precision. Therefore, several nano-based systems composed by diverse materials, and thus presenting different characteristics, have been proposed and sorted in polymeric, lipid, metal and inorganic nanocarriers (**Figure 2**). Among them, it is important to underline liposomes, polymeric nanoparticles and micelles and dendrimers.

Besides the strong demand to develop alternative therapeutic options to address unmet clinical needs, the novel nanotechnology-based platforms have although important challenges, not only for industry but also for government agencies. Efficacy and safety are evaluated on proof-of-concept studies, but the manufacturing process must be robust by identifying all its critical points and thus implementing "quality-by-design" (QbD) concept or improved process analytical technologies (PAT).

Liposomes

Liposomes consist of self-assembled lipid bilayer membranes with size ranging from 90 to 150 nm, which are formed by phospholipids and cholesterol that enclose an aqueous core (**Figure 3A**). Phospholipids are composed by hydrophilic heads and hydrophobic long tails. Thus, as previously described in several reviews, their structure allows hydrophilic molecules to be incorporated within the inner compartments, while the hydrophobic compounds will be entrapped within the hydrophobic bilayer (Sahoo and Labhasetwar, 2003; Aslan et al., 2013; Sharma et al., 2013).

The potential use of liposomes as delivery systems is based on the fact that they provide a slow and sustained release, improving the accumulation of the entrapped molecules. Also, on their ability to decrease cytotoxicity of incorporated molecules, since they modulate the biodistribution and pharmacokinetics (Khan et al., 2008; Sharma et al., 2013). Having in consideration their biocompatibility, the biodegradability and ability to cross lipid bilayers and cell membranes, liposomes have been proposed as delivery platforms for vaccines, anticancer drugs and gene therapy (Ewert et al., 2005). However, one of the major drawbacks of conventional liposomes is the short circulation time, being rapidly removed by mononuclear phagocytes of the reticular endothelial system (RES). Stealth liposomes, or long-circulating liposomes, have been developed to overcome this problem. They consist in liposomes that are sterically stabilized, presenting thus a prolonged half-life (Frank, 1993; Krishnamachari et al., 2011).

Regarding the success attained by liposomal platforms in the clinic and advanced-stage clinical trials, several liposomal-based delivery systems are nowadays offered as an anticancer strategy, such as liposomal doxorubicin, cytarabine and cisplatin (Abraham et al., 2005; Huwyler et al., 2008; Aslan et al., 2013). The use of liposomes for doxorubicin delivery prevents the damage of heart and renal healthy tissues that is usually induced by the extreme toxicity of the drug (Abraham et al., 2005). Moreover, doxorubicin has already been formulated in active targeted liposomes for breast cancer therapy, using engineered peptide ligands (Sharma et al., 2013). Other attractive approach is the use of

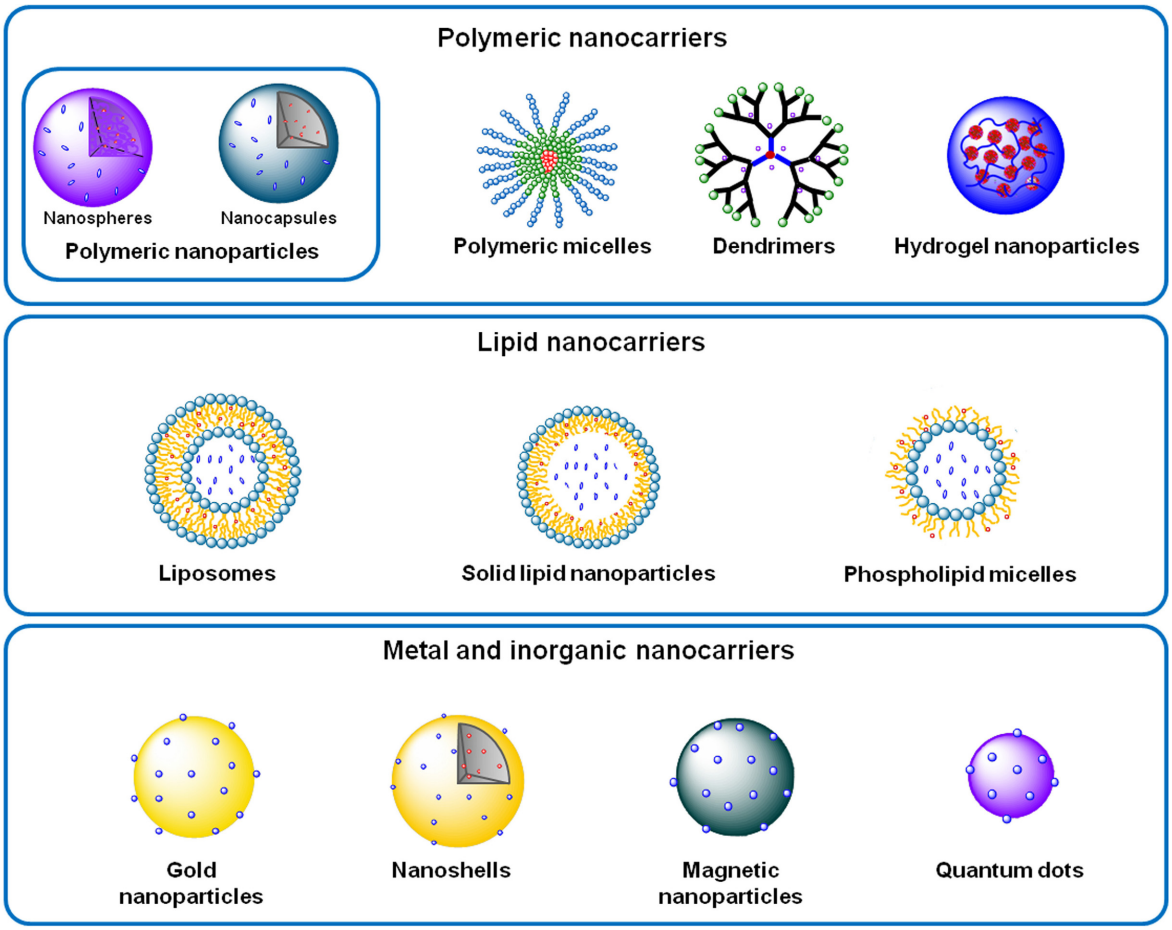

FIGURE 2 | Examples of polymeric, lipid, and metal and inorganic nanocarriers.

liposomes as carriers for antisense oligonucleotides, as siRNA, in cancer therapy (Tari et al., 1996).

Van Broekhoven et al. (2004) have reported a DC-targeting vaccine, based on a liposomal formulation, as an outstanding platform to induce a highly effective immunity against tumor cells (Van Broekhoven et al., 2004). Preclinical studies of liposome-DNA complexes have also been described, constituting an effective strategy to elicit anti-tumor immunity (U'ren et al., 2006).

The phase I clinical trial of a liposomal cancer vaccine for breast, ovarian and prostate cancer has already been reported. It has been proved that this peptide vaccine, which is intended to elicit multi-functional T-cell responses, is safe and immunogenic (Berinstein et al., 2012).

Additionally, liposomes have been studied as carriers for alternative bioorganic and biodegradable contrast agents, as glycogen and poly-L-Lysine. With these liposomes, it was possible to develop an *in vivo* multi-color magnetic resonance imaging for lymph node mapping (Chan et al., 2014).

Polymeric nanoparticles (NPs)

Polymeric NPs are submicron-sized polymeric colloidal particles with excellent features as vehicle for the delivery of drugs, biomolecules and genes (Panyam and Labhasetwar, 2003; Mahapatro and Singh, 2011).

Polymer properties such as biocompatibility, low toxicity and biodegradability have highlighted polymeric NPs as an interesting delivery strategy. The chemical structure of the polymers is easily modified, allowing the development of multifunctional engineered systems. Nanoparticle size, shape and surface properties can also be tailored, as well as the degradation kinetics and mechanical properties (Albertsson, 2002).

Polymeric NPs are usually highly stable and can easily entrap and/or adsorb both hydrophilic and hydrophobic molecules with good efficacy (Gelperina et al., 2005). The drug entrapment protects molecules from degradation (Singh and Lillard, 2009). Additionally, as nano-sized polymeric particles, these carriers are easily transported through extra and intracellular barriers. As a result, entrapped agents may be delivered site-specifically, for instance in inflamed areas or tumors, after crossing the endothelium (Prokop and Davidson, 2008; Singh and Lillard, 2009).

Two different types of polymeric NPs are usually considered: nanospheres and nanocapsules (**Figure 3B**). Nanospheres consist in a polymeric matrix in which the drug or cargo is homogenously dispersed, whereas nanocapsules are vesicular systems formed by

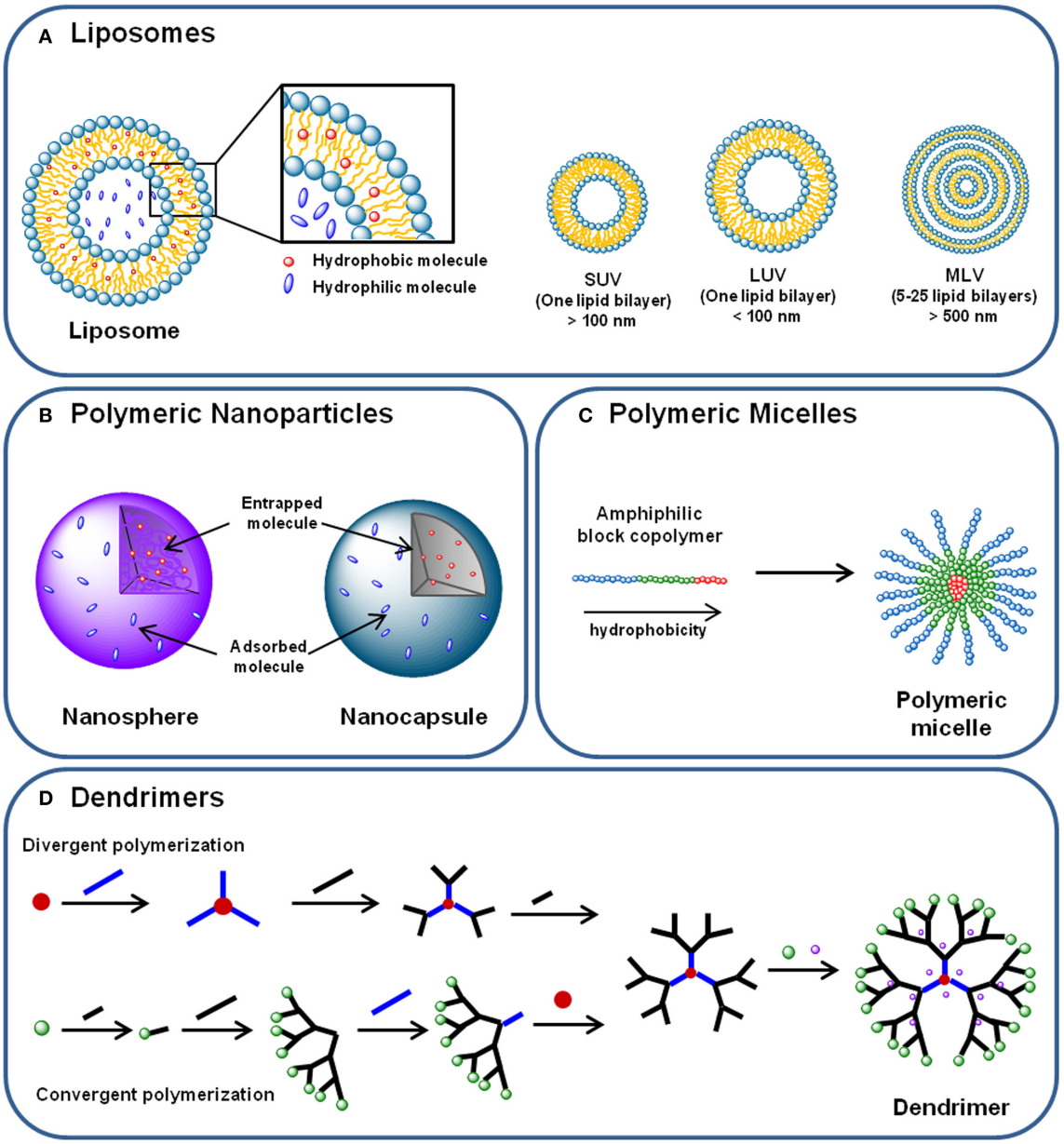

FIGURE 3 | (A) Liposomes are phospholipid and cholesterol self-assembled bilayer membranes that enclose an aqueous core, where hydrophilic molecules can be incorporated. Hydrophobic compounds can also be incorporated in the lipid bilayer. Liposomes can be classified in (i) small unilamellar vesicles (SUVs); (ii) large unilamellar vesicles (LUVs) and (iii) multilamellar vesicles (MLVs), according to their size and lamellarity. **(B)** Polymeric nanoparticles are submicron spherical entities composed by a polymeric compact net than can either constitute a polymeric matrix—in the case of nanospheres—or a polymeric wall surrounding a vesicular core—nanocapsules. Nanoparticles can transport hydrophilic and hydrophobic molecules either entrapped in the polymeric matrix or core, or adsorbed to their surface. **(C)** Polymeric micelles are self-assembled spherical nanocarriers formed by amphiphilic block copolymers. In aqueous medium, the block copolymers arrange themselves in a disposition where the most hydrophobic parts of their chains form a hydrophobic core—where hydrophobic molecules can be incorporated –, and the most hydrophilic regions of the polymer chain are displayed outoward. **(D)** Dendrimers are hyperbranched nanocarriers formed by a central core, branching monomers and functionalized peripheral groups. Dendrimer synthesis can start from the core element (divergent polymerization) or from the peripheral branching units (convergent polymerization), resulting in a structure with a hydrophilic surface and a hydrophobic central core. Molecules can be transported by dendrimers either incorporated in the core and branches, either conjugated to the terminal groups.

a polymer wall that surrounds a core containing the cargo (Singh and Lillard, 2009).

Several methods have been used to produce polymeric nanoparticles. Some of the most studied are spray-drying, salting out, nanoprecipitation and emulsion-based methods, The latter, in particular, lies on an emulsification process with the removal of organic solvents used for polymer dissolution, by extraction or evaporation. The emulsified organic drops containing

the polymer originate nanoparticles, when the organic solvent is eliminated (Lassalle and Ferreira, 2007).

Nevertheless, it is important to bear in mind that the chosen method will influence the characteristics of the obtained NPs, such as the size and the surface. Besides, it is crucial to have a great knowledge about the different experimental variables, in order to achieve the intended formulation characteristics (Gorner et al., 1999; Lassalle and Ferreira, 2007).

A large number of polymers from different origins have already been described as useful materials for polymeric NP production and used in preclinical studies. Polymers can be from natural origin, as chitosan, or synthesized, as polylactic acid and poly-lactic-co-glycolic acid (PLGA) (Krishnamachari et al., 2011; Mizrahy and Peer, 2012). Particulate adjuvants, such as PLGA and PCL NPs, have generated a lot of interest due to their biodegradability, biocompatibility and mechanical strength. (Danhier et al., 2012) has nicely reviewed the main properties and applications of PLGA-based nanocarriers. These NPs can also act as adjuvants, maintaining the antigenicity and immunogenicity of encapsulated proteins. In fact, PLGA, used for decades in humans, is the most studied polymer for vaccine formulation and it was shown to increase antibody and cellular responses to antigen-loaded PLGA NP (Johansen et al., 2000; Shen et al., 2006; Chen et al., 2014a). PCL has a great potential for developing antigen controlled release matrices by its low degradation rate, hydrophobicity, good drug permeability, *in vitro* stability and low toxicity. The adjuvant effect of PCL NPs to induce immune responses against an infectious disease was previously confirmed by several studies (Benoit et al., 1999; Florindo et al., 2008, 2009b; Labet and Thielemans, 2009). If the encapsulated antigen fails to induce DC activation, these NPs can be modified with maturation signals at their surface for direct ligand-receptor interaction, as mannose receptor is overexpressed at DCs and macrophage cell surface. Chitosan NPs, for instance, are an interesting strategy for gene delivery, namely small interfering RNA (siRNA). As chitosan is positively charged, electrostatic interactions occur with negatively charged siRNA, and thus the biomolecule is safely carried to its *in vivo* target (Aslan et al., 2013).

Nanocarriers produced using polypeptide-based polyanionic, zwitteronic and polycationic polymers (e.g., polyglutamic acid, polyarginine) have also been described (Christian et al., 2009). These are endosomolytic polymers and have been used to promote the cytosolic delivery of these biomolecules. Although clinical trials with peptide-based cancer nanovaccines have shown little success, more recent research has been developed to improve them, using novel polymeric NPs systems.

It has been reported that PLGA NPs loaded with melanoma antigens can elicit effective anti-tumor activity by CTLs *in vivo* (Zhang et al., 2011; Ma et al., 2012). DC-targeting chitosan NPs, carrying IL-12, were also used in a preclinical study. The administration of this nanovaccine in an animal model resulted in suppression of tumor growth and increased induction of apoptosis (Kim et al., 2006).

Regarding immune cell tracking, biodegradable PLGA NPs have been used in a combined multimodal imaging strategy for a DC-targeting nanovaccine. Superparamagnetic iron oxide particles and a fluorescently labeled antigens were incorporated within the same nanosystem, allowing not only the analysis and quantification of NPs uptake, but also the subcellular tracking of NPs (Cruz et al., 2011).

Polymeric micelles

Polymeric micelles are self-assembled spherical nanocarriers formed by amphiphilic block copolymers in aqueous medium (**Figure 3C**). A hydrophobic core and a hydrophilic surface compose these structures, and their size ranges from 10 to 100 nm (Torchilin, 2001; Jhaveri and Torchilin, 2014).

Polymer micelles have been investigated as delivery systems for poorly water-soluble/hydrophobic drugs due to the hydrophobic core. It has been shown that micelles can enhance the bioavailability of hydrophobic molecules, which is reassured because they protect the drug from *in vivo* degradation (Torchilin, 2001; Jhaveri and Torchilin, 2014). Other advantages of polymeric micelles are the low toxicity, the prolonged circulation time and good levels of accumulation in tumor areas (Ganta et al., 2008). In an experiment with nude mice xenograft model, PLGA-PEG polymeric micelles have shown increased tumoral uptake (Yoo and Park, 2004).

Novel pH-responsive polymer micelles formed by an N-(2-hydroxypropyl) methacrylamide corona and a propylacrylic acid (PAA)/dimethylaminoethyl methacrylate (DMAEMA)/butyl methacrylate (BMA) core have already been investigated for antigen trafficking modulation in DCs. The results showed that this nanosystem facilitates the antigen delivery to DCs in the lymph nodes and enhances CD8+ T cell responses, being thus a potential carrier for cancer vaccines (Keller et al., 2014). Also, micelles formed by DMAEMA and pyridyl disulfide ethyl methacrylate (PDSEMA), carrying both CpG ODN and protein antigens, have shown to elicit and increase cellular and humoral immune response by modulating and stimulating antigen cross-presentation, as summarized by Wilson et al. (2013).

Dendrimers

Dendrimers consist in hyperbranched spherical nanocarriers formed by a central core, branching monomers and functionalized peripheral groups. Dendrimers can be produced by convergent or divergent polymerization of branching units, resulting in a structure with a hydrophilic surface and a hydrophobic central core (**Figure 3D**) (Lee et al., 2005). Their main physicochemical features are low viscosity, hyperbranched molecular topology, marcromolecular size, high density of chemical functionality and multiple end groups that can be chemically functionalized (Lee et al., 2005). Also, the depolymerization of dendrimers can be tailored in order to control the release profile of the loaded agents, as described in a review by Wong et al. (2012). Besides vaccines, therapeutic and targeting carriers, dendrimers have also been reported as diagnostic tools due to their ability to protect imaging agents, decreasing its toxicity and enhancing specificity (Yang et al., 2009).

Nowadays, the most described family of dendrimers is the well-studied polyamidoamine (PAMAM). Poly(propyleneimine) and peptide dendrimers, such as poly(L-glutamic acid) dendrimers, have also been studied (Nanjwade et al., 2009).

Linear poly(glutamic acid) is a poly(amino acid) polymer with considerable potential for antigen delivery to DCs, and adjuvant properties for DC maturation, able to induce CTLs (Yoshikawa et al., 2008). Additionally, it has been shown to be safe for use in clinic (Chipman et al., 2006) providing the necessary safety profile for human use. These glycopeptide dendrimers have shown promise for antitumor and antiviral prophylactic or therapeutic vaccines, as well as antiviral agents (Niederhafner et al., 2008). Several formulations have reached clinical trials as vaccines against breast (Gilewski et al., 2007), prostate (Slovin et al., 2003), and small cell lung cancers (Krug et al., 2004) with encouraging results. Even though, further investigation must be done in order to guarantee the long-term safety, before they become clinically available (Aslan et al., 2013).

INFLUENCE OF NANO-BASED TECHNOLOGY PROPERTIES IN CELLULAR UPTAKE

Arguably, the weakest link in preclinical experimentation of nanodelivery systems is the continued failure to document dynamic processes (over time) using complex biosystems as models, i.e., a systems biology approach. The outcome of different classes of nanomedicines under preclinical and clinical evaluation has demonstrated that their main biological consequences of cellular or subcellular targeting and access are closely related to materials intrinsic properties (Ehmann et al., 2013).

The uptake of TAAs, carried within nano-platforms, by DCs is in fact influenced by several particulate physicochemical properties. Size, shape, surface charge, hydrophobicity and receptor interactions are generally underlined (Foged et al., 2005; Bachmann and Jennings, 2010). Particulate vaccines, such as whole-cell vaccines, virosomes, VLPs or formulated delivery platforms such as liposomes, micro and NPs have great surfaces with electrostatic or receptor-interacting properties, leading to an increased interaction when compared to soluble antigens (Bachmann and Jennings, 2010). Also, it has been reported that particulate size can direct the DC subset target. However, the ideal dimensions of NPs for APC uptake are still under discussion. In fact, small size platforms (<200 nm) may drain freely to LNs, being thus taken up by LN-resident DC subsets such as CD8α+, which seems an advantage for cancer immunotherapeutic approaches. However, delivery systems greater than 200 nm appear to be taken up by circulant monocytes, which differentiate after particle uptake and migrate to LNs afterwards (Manolova et al., 2008). According to Foged and colleagues, NP size should be 0.5 μm or less to be quickly and efficiently incorporated by DCs (Foged et al., 2005).

Size

NP size appears to influence the cellular uptake mechanism and the endocytic pathway of NPs, dictating their ultimate intracellular fate and thus overall biological effect. NPs may be assimilated by receptor-mediated endocytosis, clathrin-dependent and/or caveolae-mediated, and phagocytosis, or through a receptor independent mechanism—macropinocytosis. Particulate systems with a larger diameter (>0.5 μm) tend to be assimilated through macropinocytosis and/or phagocytosis by some specific cells, as macrophages and Langerhans cells in the skin. Smaller particles usually enter the cell through endocytosis. NPs with size <150 nm are generally taken by cells via classic receptor-mediated endocytosis (clathrin-dependent) or endocytosis caveolae-mediated if ranging from 50 to 80 nm (Pelkmans and Helenius, 2002). These NPs with size equivalent to viruses are usually able to initiate a virus-like immune response with activation of CTL and Th1. On the other hand, larger particles normally generate a similar immune response to that induced by bacteria, with Th2 activation and antibody production (Xiang et al., 2006).

Shape

Besides size, it has also been reported that particle shape may influence cellular uptake and biodistribution. Although it has been suggested non-spherical particles may be valuable for their increased blood circulation time, due to reduced phagocytosis by unspecific cells, they also demonstrated decreased cellular uptake, when compared to spherical NPs. According to Gratton et al., rod-shaped NPs show the highest uptake performance, followed by spheres, cylinders and finally cubical NPs (Gratton et al., 2008).

Surface charge

NP surface charge also seems to play an important role in their particle internalization and thus will also determine the nature of the induced immune response (Xiang et al., 2006). As cell membrane charge is negative, positively charged molecules/systems will show high affinity to it. After cellular uptake, it has been observed that negatively charged or neutral NPs tend to localize within lysosomes, whilst positively charged NPs showed ability to escape from these. Cationic NPs were found in the perinuclear area and have been reported as effective for uptake by macrophages and DCs (Thiele et al., 2003; Yue et al., 2011). On the other hand, the interaction of those delivery systems with cell depends on multiple factors and some studies have reported the presence of neutral NP at endoplasmic reticulum, suggesting their ability to escape degradation at lysossomal/endossomal compartment (Zhou et al., 2014).

NANOCARRIERS FOR TUMOR AND IMMUNE CELL TARGETING

Passive targeting

Passive targeting results from the transport of nano-based systems across the abnormal leaky vasculature of tumors, into the tumor interstitium or cells, by their movement within fluids—convection—or by passive diffusion. Whereas convection is observed for larger molecules, compounds with low molecular weight cross the membranes by diffusion, depending only on the concentration gradient (Iyer et al., 2006; Danhier et al., 2010).

As blood vessels architecture and its regulation are compromised, caused by unpaired angiogenesis, nanocarriers tend to accumulate selectively in tumor interstitium due to the "Enhanced Permeability and Retention (EPR) effect." The increased size of gaps in endothelial cells creates pores ranging from 10 to 1000 nm, which along with the poor lymphatic drainage, contributes to the EPR effect, that was first described by Matsumura and Maeda (1986); Yuan et al. (1995); Danhier et al. (2010). This effect has become very important for the design of targeted nanocarriers for cancer therapies. It has been reported that NP levels of accumulation in tumor interstitium

are 10–50-fold higher than in normal tissues, leading to improved therapeutic efficacy and less side effects (Iyer et al., 2006; Danhier et al., 2010).

Active targeting

Nanotechnology-based strategies have been explored as platforms for drug delivery, cancer vaccination and/or diagnosis, due to their capacity for overcoming biological barriers and to modulate payloads' intracellular trafficking. These nanoparticulate systems present a good potential for site-selective delivery by binding recognition ligands to NP surface, which can enhance NP endocytosis, influencing their intracellular trafficking and thus inducing prolonged effects (Danhier et al., 2010).

Surface functionalization of nano-based systems (**Figure 4**) has been used to improve tissue and cell surface antigen targeting, thus moderating non-specific distribution and prolonging the blood circulation time of nano-based systems (Alexis et al., 2008).

PEGylation is a widespread strategy to improve the half-life time of nanocarriers, through steric stabilization and "stealth" properties. It relies on the introduction of poly(ethylene glycol) (PEG) molecules by conjugation, grafting or adsorption onto the surface of nanosystems (**Figure 5**). The terminal groups of PEG chains also present very suitable moieties to attach functional ligands and attain active-targeted carriers (Freichels et al., 2012). The conjugation of antibody fragments to PEG ends, using disulfide bonds, may consist in an interesting strategy to develop platforms for active targeting (Brocchini et al., 2008). D-α-tocopheryl polyethylene glycol succinate (TPGS) has been reported as an alternative to PEG (Pan and Feng, 2008).

Active-targeted nanosystems are based on the design of nanocarriers with bioactive ligands placed onto their surface or periphery. They will be recognized by overexpressed molecular patterns at the tissues/cells intended to target, facilitating NP recognition and subsequent receptor-mediated endocytosis (**Figure 6**) (Cheng et al., 2007; Kumar et al., 2009; Danhier et al., 2010; Aslan et al., 2013; Nicolas et al., 2013; Wang et al., 2013a; Gao et al., 2014). Surface modifications represent an outstanding tool for cell targeting allowing a specific contact of nanoparticulate systems with critical immune cells, as evidenced in Stephan et al. (2010). For example, the ligand DEC-205 is highly expressed by CD8+DCs, cells particularly efficient at "cross-presenting" exogenous antigens on MHCI, constituting a highly relevant pathway for the development of a cytolytic immune response. Moreover, recent studies have indicated that the triggering of CD40 on APCs can lead to CD8 T-cell effectors, without the need of common stimulation by MHCII-related Th cells via CD40 ligands (Vonderheide et al., 2013). Mannose receptors at DCs are also associated to ligand internalization and further processing and presentation by immune cells, leading to a more extensive immune response (Lu et al., 2007; Carrillo-Conde et al., 2011; Silva et al., 2013).

These ligands, such as peptides, antibodies and antibody fragments, carbohydrates and even vitamins, may be either attached before the nanocarrier production or afterwards. Liking ligands

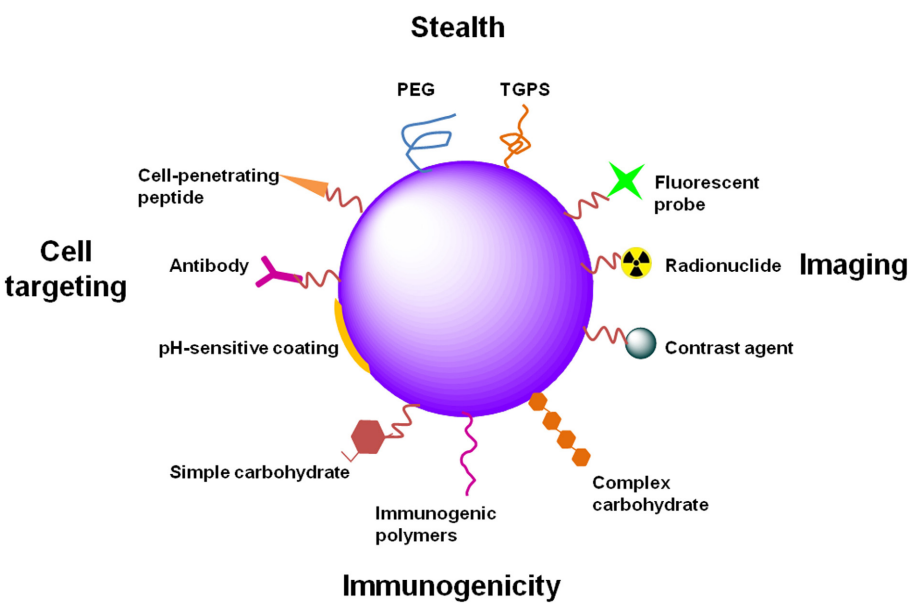

FIGURE 4 | Examples of NP functionalization. NPs can be functionalized differently in order to attain distinct goals. PEG or TGPS functionalization provide stealth properties to NPs, avoiding capture by phagocytic cells and increasing their circulation time. Functionalization of NPs with imaging agents, such as fluorescent probes, radionuclides or contrast agents (e.g., gold or magnetic NPs), provide applicability of NPs to diagnostic, theranostic or even *in vivo* real-time imaging. The immunogenicity of NPs can be increased for immunotherapy or prophylactic vaccination. Different molecules can be used for that propose, such as PAMPs (several carbohydrates, lipids or nucleic acids) or immunogenic polymers (e.g., chitosan, alginate, poloxamers). Specific tissue and cell targeting can be achieved through the functionalization of NPs with antibodies directed to specific or overexpressed antigens. Cell-penetrating peptides can improve NP internalization. pH-sensitive coatings allow drug release in specific tissues or intracellular compartments in a pH-dependent manner.

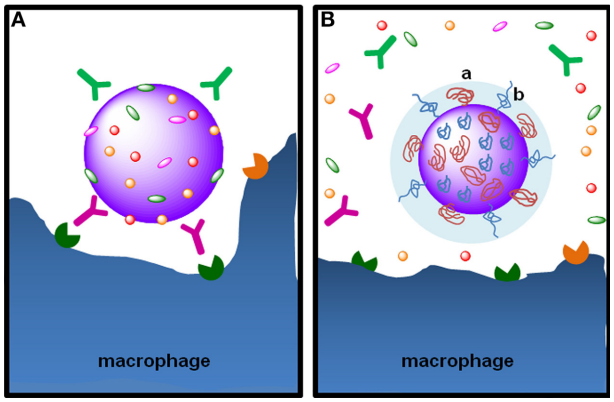

FIGURE 5 | The stealth effect from NP functionalization with PEG. (A) Particulate foreign entities in body fluids are promptly covered with opsonins, such as the immunoglobulins IgG and IgA and the complement proteins C3b C4b, in a process called opsonization. Opsonins mark the particulate entity to phagocytosis through their recognition by Fc receptors on phagocytic cells, such as macrophages. **(B)** Functionalization of NPs with PEG by grafting, conjugation or adsorption—note the "mushroom-like" (a) or "brush-like" (b) configuration of PEG chains—provides steric stabilization and stealth properties, preventing the adsorption of opsonins at the surface of nanoparticles. PEG hydrophilicity attracts water molecules to particle surface avoiding the adsorption of opsonins at NP surface, rendering them "invisible" to phagocytic cells.

prior to nanocarrier production may be advantageous, so that the conjugation yield of the ligand to the polymer can be assessed and controlled. Nanocarriers can be thus produced with a well-characterized (co)polymer and the density of ligands on their surface can be tailored. Physicochemical properties of the polymers must be evaluated after ligand conjugation, because the hydrophilic/hydrophobic balance may be altered, particularly if macromolecules are linked (Betancourt et al., 2009; Sperling and Parak, 2010; Nicolas et al., 2013).

The strategy of attaching ligand molecules after nanocarrier production is usually applied, when antibodies, proteins and polypeptides are chosen as targeting agents. As some organic solvents are generally used in the preparation of nanosystems, this method is preferred to avoid denaturation of the secondary structure of the ligands. Also, since they are bulky molecules, they will disturb the hydrophilic/hydrophobic balance which can difficult the method of nanocarrier production (Nicolas et al., 2013). The drawbacks of this approach are related with subsequent purification of the formulation and its characterization. The processes frequently used for purification, such as centrifugation, filtration and dialysis, may degrade or alter the nanosystems. Additionally, it is usually difficult to prove that the ligand is covalently linked to the surface of the nanocarrier and not only adsorbed (Nicolas et al., 2013).

Ligation strategies for functionalization. Several pathways have been developed to attach ligands onto nanosystems surface, such as the carbodiimide strategy, the Michael addition pathway, the biotin–streptavidin approach and the Copper-catalized ligation method (Betancourt et al., 2009; Nicolas et al., 2013). The native terminal groups of some polymers or specific moieties, introduced through chemical modifications, are generally used to apply these schemes of functionalization. For instance, carboxylic acid terminals in aliphatic polyesters and poly(ethylene glycol) (Betancourt et al., 2009).

The most used scheme is based on the carbodiimide chemistry. It relies on the coupling of a molecule containing a terminal amine group with another with an N-hydroxysuccinimide (NHS) ester end or an end group that can be easily esterified to NHS moiety (Betancourt et al., 2009; Nicolas et al., 2013). The Michael addition pathway is based on the thiol-maleimide coupling. Maleimide-polymers are used to produce nanocarriers, which are then decorated with thiol-containing targeting agents (Betancourt et al., 2009; Nicolas et al., 2013). However, the presence of native thiol groups in some molecules, as proteins and peptides, is usually low (or absent in some cases) and many are hard to access. To overcome this, disulfide bonds can be reduced in thiol groups or heterobifunctional cross-linking agents may be used (Nicolas et al., 2013). The biotin–streptavidin approach utilizes a strong non-covalent biological interaction between biotin and avidin (Betancourt et al., 2009; Nicolas et al., 2013). Still, for this strategy, a targeting agent is usually chemically bound to avidin, which is a bulky glycoprotein that may then obstruct the interaction ligand-receptor, essential for targeting (Betancourt et al., 2009). The Copper-catalized ligation is a highly efficient method, based on a cycloaddition reaction that fits in the "click chemistry" class of reactions. The chemical reaction is developed in mild conditions and with little or absent byproducts. The major disadvantage of this approach is the elimination of the Cu-based catalyst used for the reaction (Nicolas et al., 2013).

Functionalization of nanosystems for immune cell targeting. Extensive research has been made regarding cell surface receptors in immune cells, the so-called PRRs. PRRs recognize pathogen-associated molecular patterns (PAMPS) and are involved in several stages of the immune response, from its initiation and proliferation, to its execution (Kumar et al., 2009). Different types of molecules may act as PAMPs, known as "danger signals," for instance lipids, lipoproteins, proteins, carbohydrates and nucleic acids. The recognition of PAMPs by PRRs triggers immune responses by activating multifactorial signaling pathways. This leads to the induction of inflammatory responses mediated by several cytokines and chemokines (Kumar et al., 2009).

Several classes of PRRs have been reviewed, such as TLRs, retinoic-acid inducible gene (RIG)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), DNA receptors (cytosolic sensors for DNA), scavenger receptors, and C-type lectin receptors (CLRs) (Kumar et al., 2009; Carrillo-Conde et al., 2011; Shen et al., 2013; Silva et al., 2013). In mammals, the most studied PRR class is the TLRs class. TLRs are predominantly expressed by APCs, as DCs, but they are also found on cells of the adaptive immune system, such as in αβT cells, regulatory T cells, and γδT cells, as well as NKT cells (Wesch et al., 2011). Through TLR activation, both the innate and the adaptive immune responses can be engaged, either by direct activation of TLRs with their ligands on T and B cells, or by

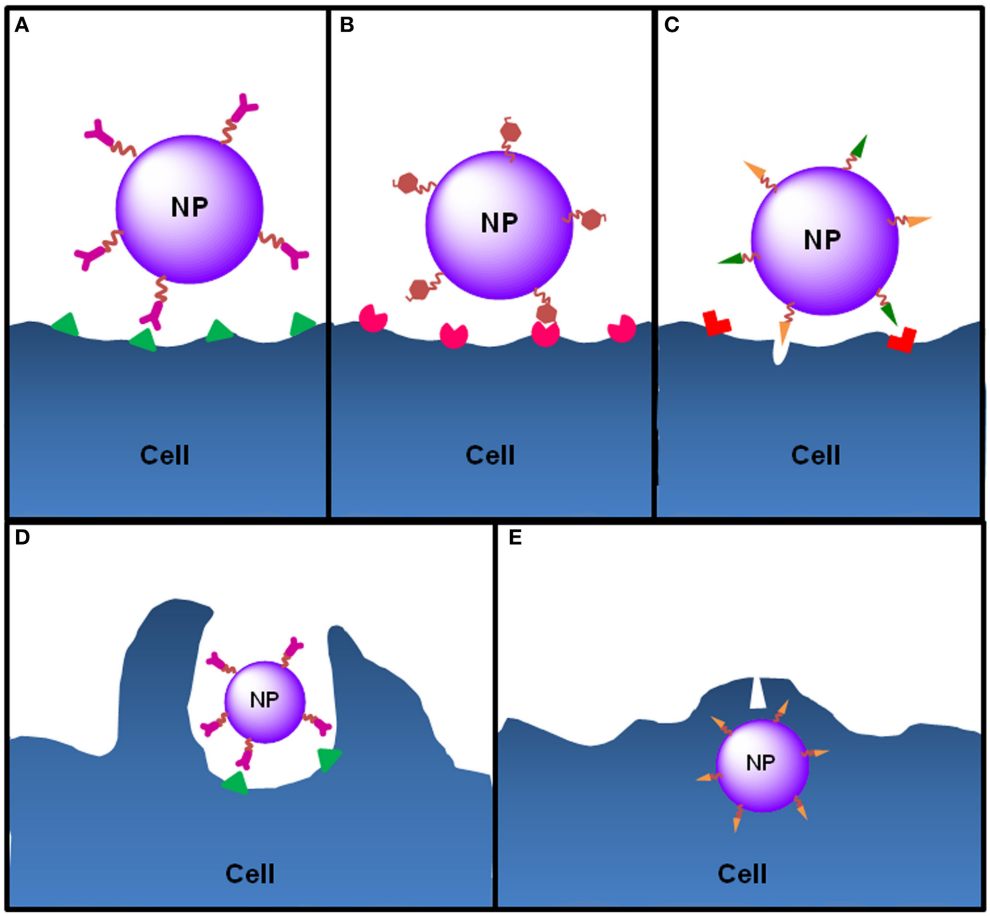

FIGURE 6 | Ligand-cell interaction and NP internalization. NPs can be functionalized with different ligands to increase cell targeting and NP internalization. **(A)** Functionalization of NPs with antibodies allows the targeting of antigens exclusively expressed or overexpressed by target cells (e.g., anti-CD205 antibody to target CD205 on DCs or anti-HER2 antibody to target HER2 on breast cancer cells). **(B)** In order to target DCs, NPs can be functionalized with molecules that mimic PAMPs, normally carbohydrates, nucleic acids or lipids, which are recognized by PRRs expressed by DCs. For instance, mannose or fucose residues are recognized by the mannose receptors—a C-lectin receptor. Bacterial lipopolysaccharide or flagellin target TLR4 and TLR5 on DCs, respectively. **(C)** Cell-penetrating peptides are small amino acid sequences normally used by viruses or bacteria to facilitate cellular invasion by those pathogens and can be used to increase the internalization of NPs. Functionalized NPs see their internalization by target cells increased essentially by two mechanisms: induction of endocytosis upon ligand-receptor binding, which happens to NPs functionalized with ligands such as antibodies, PAMPs or some penetrating peptides that induce receptor-mediated endocytosis (e.g., integrins) or **(D)** through direct cell penetration across the plasma membrane (e.g., antimicrobial peptides or histidine-rich peptides) **(E)** or both (e.g., HIV TAT peptide).

indirect mechanisms involving TLR-activated DCs (Silva et al., 2013). C-type lectin receptors (CLRs) belong to another class of PRRs expressed by APCs. This receptor family is characterized by the presence of domains that bind to carbohydrates (Van Kooyk, 2008). CLRs are specific receptors particularly engaged in the internalization of antigens. CLRs enable the intracellular uptake and processing of antigens, as well as influence their cytosolic fate and the loading on MHC class I and II (Unger and Van Kooyk, 2011).

Regarding the involvement of PRRs in several strategic immune pathways, the design of nano-based systems for immune cell targeting can be extremely interesting. Not only because a more specific delivery can be achieved, but also because the cellular internalization of the targeted nanosystem can be modulated and potentiated. Additionally, the attachment of PRRs ligands on the surface of nanocarriers may boost their immunogenicity, which can be an outstanding strategy for the development of vaccines, since it allows the incorporation of an antigen and the "danger signal" in the same platform (Silva et al., 2013).

NANOCARRIERS FOR IMAGING APPROACHES

The importance of a deeper knowledge of the dynamic cancer immunological processes has long been realized. The study of these processes *in vivo*, with living cells and the whole organism, is essential to answer this issue more accurately. Cancer disease processes will be better understood and thus improved therapies can surely be developed. For the visualization of these biological dynamic processes *in vivo*, methods have to provide a real-time *in situ* fast response, as well as be non-invasive and with high sensitivity and stability (Wang et al., 2013b).

The use of targeted nanoplatforms for this purpose enables a more specific interaction with the intended target, with minimal interference to the biological system (Ballou et al., 2004). Additionally, nanocarriers may be functionalized with single or multiple ligands, which may be important for the design of complex experiments. The targeting of ligands may enhance the selective recognition of the nanodelivery systems by cells, facilitating their endocytosis. This will allow nanosystems to be used as non-invasive localization, monitoring and assessment platforms, for instance, for site-specific intracellular characterizations and real-time tracking (Ruan et al., 2007).

Fluorescence imaging techniques

Fluorescence imaging is an optical imaging method based on the excitation/emission of molecules (Cai and Chen, 2007). The use of fluorescent molecular probes—as fluorescent dyes and fluorescent proteins—has been widely applied in the labeling of biomolecules, cells and tissues. Although these probes are already used *in vivo*, for instance in retinal angiography and visualization of arteries, they are unsuitable for real-time imaging assays, regarding their low photostability and sensitivity at the cellular and molecular levels (Santra and Malhotra, 2011). The application of fluorophores in real-time *in vivo* imaging has also been limited by the high absorption of optical signal by tissues and body fluids in the UV and visible wavelength. The light scattering caused by tissues that attenuate the optical signal and the tissue auto-fluorescence that influences the background signal is also a limitation (Santra and Malhotra, 2011). Additionally, some fluorescent probes may be toxic for cells and body (Li et al., 2013). Several NP-based strategies have been proposed to overcome the limitations of fluorescent dyes for real-time *in vivo* imaging (Supplementary Material) (Santra and Malhotra, 2011; Wang et al., 2013b).

Fluorescent-labeled NPs are more stable in the body and increase the detection sensitivity and photostability. In the same platforms, a great number of probe molecules can be incorporated, in opposite to a single conventional molecule. Also, in NPs, fluorescent dyes can be protected from quenching and degradation (Santra and Malhotra, 2011; Wang et al., 2013b).

The most extensively studied nanosystems for fluorescence imaging are quantum dots (QDs) (Cai and Chen, 2007), inorganic fluorescent NPs that can be based on metallic or semiconductor materials, such as CdSe and CdTe (Ballou et al., 2004). As reviewed by Cai and Chen, in ideal conditions, QDs can have better properties than organic fluorescence probes. These include high resistance to degradation and photobleaching, high quantum yields, high molar extinction coefficients, continuous absorption spectra covering from UV to near-infrared, long fluorescence lifetimes (>10 ns), narrow emission spectra and very long effective Stokes shifts (Cai and Chen, 2007). QDs have been used for innumerous applications, from cell tracking (Voura et al., 2004) to mapping of sentinel lymph nodes (Ballou et al., 2007). QDs can be used to identify several ligands in the same experiment, using multiple colors and intensities to detect different structures (Ballou et al., 2004). The potential use of DC-targeting QDs as both fluorescent NPs for *in vivo* and *in vitro* imaging, and antigen-delivery system has also been investigated. In this study, it was proved that QDs display promising properties for combined priming and immunoimaging of DC (Sen et al., 2008). Functionalization and modifications of the surface of QDs with PEG chains and ligands for active targeting, such as peptides and antibodies, have been under research to improve the application of these nanosystems in the biomedical field (Ballou et al., 2007; Cai and Chen, 2007). QD conjugates are already commercially available for immunospecific labeling (Ballou et al., 2004). Thus, the development of multifunctional nanoplatforms holds a great promise for the future of biomedicine, since it will be possible to combine simultaneously both diagnosis and therapy in the same nanostructure (Kim et al., 2008a).

Several other groups have suggested the use of silica-based NPs (siNPs) as an interesting strategy to perform imaging assays using fluorescence (Santra et al., 2005; Kim et al., 2008a; Wang et al., 2013b). siNPs have been used for high sensitive and specific *in situ* labeling and tracking of cell surface receptors (He et al., 2004, 2007). Relying on the affinity of antigen-antibody or ligand-receptor interactions, NPs were functionalized with antibodies and ligands and applied as an immunediagnostic method (He et al., 2002). siNPs have also been used as a non-invasive tool for intracellular labeling, tracking and sensing in living cells, contributing with novel information about dynamic biological processes of subcellular structures, such as lysosomes and endosomes (Shi et al., 2010). Finally, siNPs were applied to better understand the biodistribution and fate of NPs, *in vivo* (Wang et al., 2013b).

Molecular imaging techniques

The key role of immune cells in the development of future immunotherapeutic approaches against chronic pathologies, mainly cancer diseases, has fostered the design and optimization of different real-time imaging techniques, avoiding the classic *ex vivo* histologic analysis (Kircher et al., 2011; Ahrens and Bulte, 2013; Liu and Li, 2014). In fact, most of the information obtained for immune cell tracking has arisen from optical and confocal microscopy and flow cytometry. Two-photon microscopy allowed the observation of different immune cells in their biological environment at real time (Progatzky et al., 2013). However, despite being a powerful tool to observe these highly motile cells and characterize their interaction with native environment, this imaging technique is unsuitable for detection of deeper events due to tissue opacity (Dzhagalov et al., 2012).

Bioluminescence imaging techniques, on the other hand, enable deeper tissue penetrations while tracking immune cells *in vivo*. Even though, it is one of the most commonly used techniques for immune cell tracking *in vivo*, allowing whole-body non-invasive tomography. This technique is only useful for preclinical studies in small animals, due to the limits related to the attenuation of light in tissues (Kircher et al., 2011).

All near-infrared (NIR) multiphoton microscopy methods are potential techniques for deep tissue imaging but further studies are needed to better characterize the capabilities of these NIR-excitation techniques and background reduction (Joshi et al., 2013).

Magnetic resonance imaging (MRI), ultrasound, positron emission tomography (PET) (Yaghoubi et al., 2009), single

photon emission tomography (SPECT) and X-ray computed tomography (CT) are the imaging techniques approved for medical applications (Bernsen et al., 2014). PET and SPECT are high-sensitivity and low-resolution techniques, while MRI and CT provide high-resolution images (Liu and Li, 2014). However, the use of radionuclide-based techniques, as PET and SPECT, has brought questions regarding their safety (Laskey et al., 2010). In addition, their combination with additional methods is fundamental to obtain an anatomical image. Therefore, the combination of these different imaging modalities constitutes a multimodality imaging method that has been explored in preclinical and clinical development, including SPECT/CT and MRI/PET (Naumova et al., 2014).

Among these techniques, MRI is the most versatile and sensitive method allowing the study of immune cell morphology and function (Ahrens and Bulte, 2013). In fact, innovative and safer techniques are emerging from the use of different biocompatible cell labeling probes and MRI to obtain high-resolution images without using ionizing radiation (Sosnovik and Nahrendorf, 2012; Thu et al., 2012). The signal used for MRI arises from the water protons (1H) or different fluorinated molecules (e.g., ^{19}F) under a static magnetic field and after pulsed by a radio-frequency radiation, which alters the equilibrium of their nuclei. The MRI signal will then result from a transient voltage determined by the properties of labeled tissue (Ahrens and Bulte, 2013).

This non-invasive and safe imaging technique has been expected to track immune cells *in vivo*, enabling the characterization of their biodistribution and fate. MRI also seems suitable for the detection/quantification of surface markers and secreted factors resultant from biological processes occurred *in vivo* at a particular disease stage (Lu et al., 2013; Naumova et al., 2014). The rapid evolution in this field, advanced by the potential efficacy of next-generation cellular-based therapeutic approaches (e.g., immunotherapy and stem cell-based therapy), will certainly make this method a crucial tool to follow detailed biological and immunological processes *in vivo*.

The successful application of these *in vivo* cell-tracking tools can potentially optimize image-guided diagnostics and the overall efficacy of different therapeutic options. Particularly, those based on the modulation of endogenous cells support the selection of a specific treatment, the choice of the best administration route and also the use of a correct dose for each patient (Ahrens and Bulte, 2013).

Different exogenous cell-labeling probes have been explored but superparamagnetic iron oxide (SPIO) nanoparticles and perfluorocarbon (PFC) nanoemulsions seem to be the most promising for those advanced MRI-based techniques (Supplementary Material). Moreover, these are the unique *in vivo* MRI cell-labeling techniques approved for human clinical trials, and thus will be further discussed (Ahrens and Bulte, 2013).

Nano-based systems for MRI real-time tracking of immune cells. Different nanosystems (Supplementary Material) have been developed for MRI-based *in vivo* cell tracking, but the negative contrast agents based on SPIO and PFC constitute the most explored ways to control MRI signal and consequent detection (Hawrylak et al., 1993; Bulte and Kraitchman, 2004). SPIO contrast agents are small particles composed by ferrous and ferric oxides, usually coated by dextran. Even though, these ionic NPs have been modified by other biodegradable polymer (e.g., chitosan, PEG, siloxanes, polyaniline, glyceryl monooleate) and labeled with targeting moieties to potentiate their delivery to certain tissues (Supplementary Material) (Shubayev et al., 2009; Dilnawaz et al., 2010). These MRI-based contrast agents strongly perturb the magnetic field of the region in which they are embedded. The water molecules will sense that alteration in the magnetic field and the resultant loss of signal will lead to a dark image (Ahrens and Bulte, 2013). On the other hand, fluorinated-based probes directly label targeted cells and thus the MRI signal is dependent on the number of fluorine atoms and labeled cells, which can be observed in their biological environment (Srinivas et al., 2012).

The labeling of cells using these nano-based systems can be performed *ex vivo* or *in vivo*, through their direct administration in the body. The labeling of immune cells *ex vivo* with SPIO NPs has been explored to track and clarify migratory patterns of diverse immune cells, as NK (Daldrup-Link et al., 2005), cells from T lineage (Kircher et al., 2011), and DCs (De Vries et al., 2005; Rohani et al., 2011) used during immunotherapeutic cancer approaches. Innovative immunotheranostic strategies under development combine these metal ion-based NP with targeted nanoparticulate cancer vaccines. One interesting study has shown multifunctional iron oxide NPs formulated in order to deliver carcinoembryonic antigens to DCs and be detected by MRI (Cho et al., 2011). Alternatively, some SPIO NPs have been developed to label DCs membranes by modifying their surface with CD11c antibodies, promoting receptor-mediated endocytosis (Ahrens et al., 2003; Yu et al., 2012). Despite being a promising approach against cancer disease, their clinical translation is still unclear.

The *ex vivo* labeling of a DC-based cancer vaccine by SPIO NP was used in the first clinical trial that involved the cell tracking by MRI techniques, where it was possible to detect the target lymph node only in half of the patients with melanoma (De Vries et al., 2005).

T cells have been sorted and cultured with SPIO NPs, mostly coated by transfection agents, as poly-L-lysine or protamine sulfate, to promote their capture due to the non-phagocytic nature of these immune cells (Arbab et al., 2005; Thorek and Tsourkas, 2008; Thu et al., 2012). These intracellular labeling was also attempted through the use cell-penetrating peptides and HIV-TAT (Torchilin, 2008).

The *in vivo* labeling of immune cells by SPIO NPs is often used to track monocytes and macrophages to characterize inflammatory events, due to their phagocytic behavior (Settles et al., 2011). The *in vivo* labeling can be achieved by the intravenous administration of SPIO NPs, or alternatively after their direct injection into tumor tissue. Both options were successfully used to label immune cells and track their migration pattern toward lymph nodes, which allows for example the definition of tumor specific stage (Harisinghani et al., 2003).

It is important to emphasize that the cell labeling strategy must not alter the function and normal phenotype of immune cells, which could limit the efficacy of cellular-based therapies.

The SPIO NPs are known as safe systems due to their biodegradability nature and usual rapid metabolization *in vivo* (Yu et al., 2012). Therefore, the SPIO-based cell labeling is mostly suitable for short-term studies. On the other hand, false positives may be detected after the accumulation of the detection agent in macrophages after the destruction of labeled cells (Ahrens et al., 2003; Thorek and Tsourkas, 2008). This disadvantage is in fact common to different imaging reagent-labeled techniques.

The ^{19}F MRI is a highly sensitive technique that allows the direct quantification of labeled immune cells, as T cells and phagocytic cells, either *in vivo* or *ex vivo* (Srinivas et al., 2009; Helfer et al., 2010). Unlike SPIO NPs, this labeling method usually does not detect false positives and, once is not metabolized *in vivo*, constitutes a suitable approach for long-term studies (Janjic and Ahrens, 2009; Srinivas et al., 2012).

The droplet surface of these PFC colloidal systems has been changed with charged entities to potentiate their efficient delivery at intracellular level. Therefore, the safety of these labeling systems is increased, which has been shown using different immune cells, as DCs and T cells (Ahrens et al., 2005; Srinivas et al., 2009; Helfer et al., 2010; Ahrens and Bulte, 2013).

Recent studies have shown the promising combination of ^{19}F labeling techniques with fluorescence or NIR probes, as well as with nuclear magnetic resonance (NMR) (Patel et al., 2013). Even though, the use of these colloidal system for cell tracking is considerably recent and further studies are urged in order to confirm these indications.

ANIMAL MODELS FOR THE TRANSLATION OF IMMUNOTHERAPEUTIC APPROACHES

The successful translation of alternative immune-based approaches for cancer therapy into the clinic is highly dependent on the development of preclinical animal models that adequately mimic human disease progression. Several models have been developed and successfully used to study cancer mechanisms of disease and the efficacy of conventional therapeutic options (Budhu et al., 2014).

Accordingly, models currently used to evaluate therapeutic antitumor efficacy at preclinical level are based on transgenic systems and the transplantation of *in vitro* grown cancer cells into healthy animals or in humanized mouse models—human tumor xenograft models (Ostrand-Rosenberg, 2004). The implantation of human cell lines dictates the use of immunocompromised mice –T-cell deficient—to allow the establishment of cancer disease. Besides being one of the most used models to study cancer disease and the effect of cytotoxic therapies, those are definitely not suitable to test the efficacy of immunotherapeutic strategies as it is not possible to evaluate the effect of adaptive immune response in tumor development (Legrand et al., 2009). However, different approaches are currently being explored to improve their application toward the reconstitution of the immune system using human cells (Carpenito et al., 2009; Legrand et al., 2009; Pedroza-Gonzalez et al., 2011). Still, the evaluation of the outcome of different immunotherapeutic options has been possible due to development of different mouse cancer cell lines, which can be further modified if needed: B16 melanoma, CT26 colon carcinoma, TRAMP (transgenic adenocarcinoma of the mouse prostate model) prostate cancer, 4T1 breast cancer, EL4 T lymphoma (Greenberg et al., 1995). Even tough, there is usually a rapid tumor growth after the subcutaneous administration of those cells and therefore these models do not mimic the long-lasting host-tumor interactions resultant from the spontaneous implementation of this disease. On the other hand, the transplantable tumors are very versatile for prophylactic studies as it allows establishment of different vaccination settings, allowing an immune response before the induction of cancer disease and consequent immunosuppressive outcomes.

The spontaneous and multi-step tumor development, including the cross-talk between cells within tumor microenvironment is possible in genetic modified animals (Dougan et al., 2011). However, these animals need to be evaluated for longer periods of time. In addition, the presence of mutations in a permanent manner, in contrast to what happens in cancer disease, has been associated with higher variability and tolerance and consequently, lower effectiveness of different immunotherapeutic options (Hurwitz et al., 2000; Ercolini et al., 2005).

As a result, there is an urgent need for animal models recapitulating cancer disease, and all results should be discussed having in consideration animal model specificities and limitations. In addition, different types of animal models should be tested in order to better characterize the obtained antitumor evidences for clinical translation.

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite the improvement observed in chemotherapy and radiotherapy for cancer treatment, the battle against this disease seems to have more chances to be achieved through the combination of different therapeutic modalities. Immunotherapeutic approaches have emerging as promising tools to address the heterogeneity of this disease, namely those immune cell mediated cancer therapies. It is possible to underline the advances obtained with the approval of anti-CTL4 monoclonal antibody by the FDA, and great expectations have arisen from the use of different approaches to modulate the function of immune cells within tumor site. Among those strategies, the outcome of cancer vaccines can be highlighted. To monitor and guide the development of cellular therapies and the *in situ* manipulation of immune cells, the improvement of non-invasive imaging strategies to obtain detailed information regarding the biological processes within the complex tumor microenvironment is imperative. We foresee the use of non-toxic nanotechnology-based systems able to combine the specific (i) targeting of immune cells, promoting the controlled delivery of different molecular entities to modulate the cell-cell interactions; and (ii) tracking through the inclusion of different probes to improve safety, specificity and sensitivity of cell-labeling methods and imaging approaches. These immunotheranostics are expected to enable a rational definition of treatment plans for a particular patient, resulting in better clinical outcomes and enhanced control of the disease, which can also promote their translation into marketed systems.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fchem.2014.00105/abstract

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Biomolecular corona on nanoparticles: a survey of recent literature and its implications in targeted drug delivery

Ryan M. Pearson[1], Vanessa V. Juettner[1] and Seungpyo Hong[1,2]*

[1] *Department of Biopharmaceutical Sciences, University of Illinois at Chicago, Chicago, IL, USA*
[2] *Department of Bioengineering, University of Illinois at Chicago, Chicago, IL, USA*

Edited by:
João Conde, Massachusetts Institute of Technology, USA

Reviewed by:
Jose Maria Montenegro Martos, University of Marburg, Germany
Kenneth A. Dawson, University College Dublin, Ireland

***Correspondence:**
Seungpyo Hong, Department of Biopharmaceutical Sciences, University of Illinois at Chicago, 833 S. Wood St., Rm 335, Chicago, IL 60612, USA
e-mail: sphong@uic.edu

Achieving controlled cellular responses of nanoparticles (NP) is critical for the successful development and translation of NP-based drug delivery systems. However, precise control over the physicochemical and biological properties of NPs could become convoluted, diminished, or completely lost as a result of the adsorption of biomolecules to their surfaces. Characterization of the formation of the "biomolecular" corona has thus received increased attention due to its impact on NP and protein structure as well as its negative effect on NP-based targeted drug delivery. This review presents a concise survey of the recent literature concerning the importance of the NP-biomolecule corona and how it can be utilized to improve the *in vivo* efficacy of targeted delivery systems.

Keywords: nanoparticle, biomolecular corona, targeted drug delivery, *in vivo* efficacy

INTRODUCTION

Medical applications of nanoparticles (NPs) are wide-reaching as evidenced by their rapid development as therapeutic and diagnostic agents (Peer et al., 2007; Zhang et al., 2008; Hubbell and Langer, 2013). In particular, significant advances have been made in cancer therapy by pursuing NPs as drug delivery systems (Gu et al., 2007; Pearson et al., 2012; van der Meel et al., 2013), however, many challenges, especially with regard to achieving precise control over nano-bio interactions, still remain to be addressed (Chauhan and Jain, 2013; Pearson et al., 2014). As increasingly more complex NP formulations move toward later stages of clinical development, the need to understand and overcome those challenges is becoming imminent.

One of the most important challenges affecting NP-based drug delivery is the formation of the "biomolecule" or "protein" corona (Cedervall et al., 2007). As NPs enter physiological fluids, proteins and other biomolecules such as lipids adsorb to their surfaces with various exchange rates leading to the formation of the biomolecular corona (**Figure 1A**) (Nel et al., 2009; Monopoli et al., 2012; Saptarshi et al., 2013). As a consequence, the "synthetic identity" of the NP is lost and a distinct "biological identity" is acquired. This new identity governs how the NP is "seen" by cells and subsequently alters the way in which NPs interact with cells. The composition of the biomolecular corona is dynamic and is highly dependent on the initial biological environment, indicating the possibility of exposure memory (Milani et al., 2012). Opsonin adsorption such as immunoglobulin (IgG), complement, and others contribute to the deteriorated *in vivo* properties of NPs by promoting immune system recognition and rapid clearance from circulation. In contrast, dysopsonins such as albumin can coat NP surfaces and enhance their biological properties by reducing complement activation, increasing blood circulation time, and reducing toxicity (Peng et al., 2013). The binding of lipids and other lipoproteins to NP surfaces can alter the uptake and transport of NPs (Hellstrand et al., 2009). Taking these observations into consideration, the concept of the personalized biomolecular corona has arisen, suggesting that NP coronas should be characterized in a disease specific manner and not merely based on generalizations obtained from the literature (Hajipour et al., 2014).

While biomolecule adsorption alters many physicochemical properties of the NP such as size, shape, surface composition, and aggregation state, NPs may also induce conformational changes to the secondary structure of adsorbed proteins altering their biological activities (Monopoli et al., 2012). In many cases, protein adsorption to NPs can induce fibrillation, immunosensitivity, and misfolding, substantially altering properties such as biodistribution and circulation half-life, cellular uptake, intracellular localization, tumor accumulation, and toxicity (Linse et al., 2007; Aggarwal et al., 2009; Karmali and Simberg, 2011). Conversely, cases have demonstrated that biomolecule adsorption serves to protect the body from the toxicity of bare NPs, facilitating receptor-mediated interactions, and improving pharmacokinetic profiles, which demonstrates its potential advantages (Peng et al., 2013).

Fundamental forces including electrostatic interactions, hydrogen bonding, hydrophobic interactions, and charge-transfer drive the association of biomolecules to the surface of NPs (Nel et al., 2009). A recent report by Tenzer et al., found that the biomolecular corona forms almost instantaneously (in less than 30 s) and is comprised of almost 300 different proteins, although it typically consists of a similar set of proteins in various quantities (Tenzer et al., 2013). However, it has been suggested that NPs cannot accommodate as many proteins on their surfaces and a significantly lower number of proteins are present because current analyses are performed over large numbers of NPs and represent macroscopic averages of protein composition (Monopoli et al., 2012). The "hard" corona is the first layer of

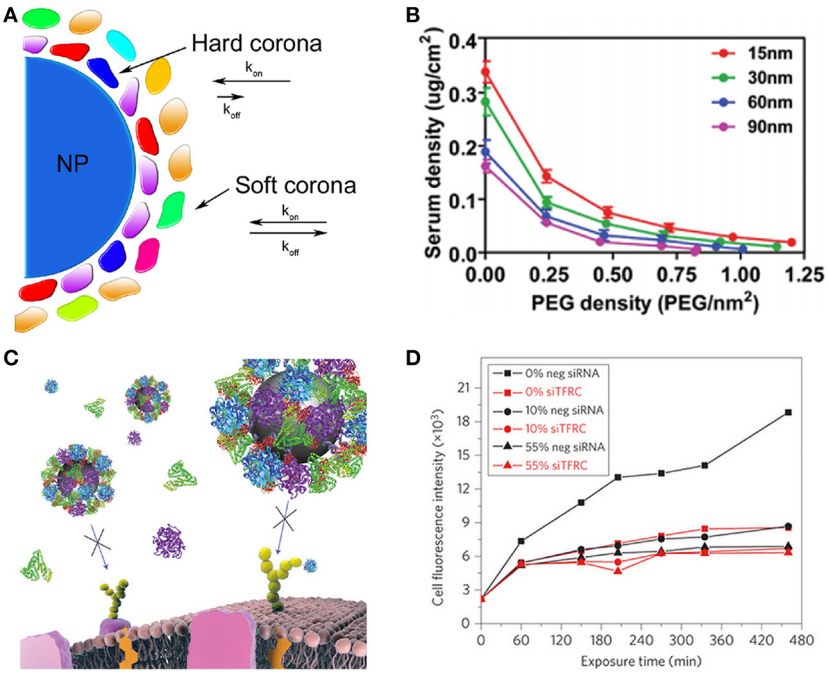

FIGURE 1 | (A) Formation of the NP-biomolecule corona. Upon exposure to physiological fluids, NPs become coated with a variety of proteins and other biomolecules. The hard corona is comprised of lower abundance, high affinity biomolecules with almost negligible exchange rates. The soft corona is comprised of more abundant, lesser affinity biomolecules with faster exchange rates. **(B)** Size and poly(ethylene glycol) (PEG) grafting density determine PEG conformation and total serum protein adsorption to AuNPs. Reprinted with permission from Walkey et al. (2011). Copyright (2011) American Chemical Society. Negative effect of the biomolecular corona on the targeted interactions of silica NPs. **(C)** Schematic of blocked targeted cellular interactions of transferrin (Tf)-targeted NP in the presence of serum proteins. **(D)** Median A549 cell fluorescence intensity of Tf-targeted NPs in various concentrations of FBS. **(C,D)** Reprinted by permission from Macmillan Publishers Ltd: [Nature Nanotechnology] (Salvati et al., 2013), copyright 2013.

the corona, consisting of tightly and nearly irreversibly bound biomolecules. Atop the hard corona lie the "soft" corona layers that are composed of more leniently associated biomolecules classified by rapid exchange rates. With increasing time, less abundant, less mobile, and higher binding affinity proteins will subsequently replace the highly abundant, lower affinity proteins (Vroman effect) (Vroman et al., 1980). However, a recent study questioned the applicability of the Vroman effect to NPs and found that the composition of the hard corona was constant over time although the total amount of adsorbed proteins was changed (Tenzer et al., 2013). Properties of NPs such as size and surface hydrophobicity have also been demonstrated to affect the composition and exchange rates of proteins such as transferrin (Tf) and albumin (Ashby et al., 2014).

Although the formation of the biomolecular corona is unavoidable and plays a significant role in determining the biological behaviors of NPs, its importance has only recently received significant scientific attention. This mini review describes the importance of the NP-biomolecule corona on determining biological responses, supported by a number of recently published reports. We will succinctly cover important aspects related to biomolecular corona formation, how it is influenced by various physicochemical properties of NPs, the impact of NPs on the structure of proteins, and the impact of the biomolecular corona on the biological interactions of NPs.

PHYSICOCHEMICAL PROPERTIES OF NPs AND THEIR EFFECT ON BIOMOLECULAR CORONA FORMATION

The physicochemical properties of NPs determine the type of corona formed. Since the interactions between NPs and proteins occur at an interface, surface characteristics of NPs ultimately drive NP-biomolecule association. To better understand corona formation, many methods have been established (Monopoli et al., 2013; Bertoli et al., 2014). Using a bioinformatics-inspired approach, Walkey et al., developed a protein corona fingerprint model that accounts for 64 different parameters to predict the cellular interactions of NPs (Walkey et al., 2014). This model was found to be 50% more accurate than pre-existing models that only consider size, aggregation state, and surface charge. Many material properties act in concert to drive biomolecular corona formation, in this section we will focus on the effect of size, surface charge, and hydrophobicity.

It is generally accepted that a positive correlation exists for NP size and protein association. For example, a two-fold increase in protein association was measured for 110 nm silver NPs (AgNPs), compared to 20 nm AgNPs (Shannahan et al., 2013). However, an inverse correlation was also reported between the amount of mouse serum protein adsorbed and the size of 5, 15, and 80 nm AuNPs (Martin et al., 2013). It was suggested that differences in curvature enabled a larger number of hydrophobic proteins to bind to the smaller NPs in this case.

Recent reports have supported the correlation between surface charge of NPs and biomolecule association. Poly(vinyl alcohol)-coated superparamagnetic iron oxide NPs (SPIONs) with negative and neutral surface charges adsorbed more serum proteins than dextran-coated SPIONs, leading to increased circulation times (Sakulkhu et al., 2014a). Biomolecule association to PSt NPs with different sizes (50 and 100 nm) and three different surface charges [charge neutral (plain), negatively charged (carboxyl-modified), and positively charged (amine-modified)] were studied to elucidate the effect of size and surface charge of NPs on protein adsorption (Lundqvist et al., 2008). A size dependency in biomolecular corona composition was observed for both types of charged PSt NPs. For example, 100 nm negatively charged PSt NPs displayed a higher fraction of unique proteins, including Ig mu chain C region, apolipoprotein L1, and complement C1q, present in their coronas, as demonstrated by low homology in biomolecule composition compared to similar 50 nm NPs.

The connection between NP hydrophobicity and protein association has been also demonstrated to be of great importance. Isothermal titration calorimetry was used to assess the stoichiometry, affinity, and enthalpy of NP-protein interactions (Cedervall et al., 2007; Lindman et al., 2007). Titration of human serum albumin into solutions of NPs comprised of different compositions of N-isopropylacrylamine (NIPAM): N-tert-butylacrylamide (BAM), it was found that more hydrophobic NPs (50:50) bound higher numbers of albumin than more hydrophilic NPs (85:15). Larger NPs bound more albumins than smaller NP counterparts. Importantly, it was also shown that apolipoprotein A-I association was 50-fold greater for 50:50 NPs than 65:35 NPs, demonstrating favorable interactions of the proteins with the hydrophobic NPs.

Although correlations have been found with those properties, it should be noted that they could only act as predictive indicators of biomolecule association to NPs. This is important since the composition of biomolecules associated with NPs *in vitro* has been shown to be different than *in vivo* (Sakulkhu et al., 2014b). Nonetheless, the findings suggested that the surface properties of NP are responsible for driving biomolecule adsorption to the NP. Therefore, to further realize the potential of NPs as drug delivery vehicles, it is critical to coat their surface with a non-fouling layer, e.g., poly(ethylene glycol) (PEG), polyoxazoline, poly(vinyl alcohol), or polyglycerol, to minimize biomolecule association and therefore achieve more controllable cellular responses (Owens and Peppas, 2006; Romberg et al., 2008; Amoozgar and Yeo, 2012).

IMPACT OF PEG LAYERS ON BIOMOLECULAR CORONA FORMATION

Modification of the surface of NPs with a layer of PEG, or PEGylation, is known to reduce opsonization and enhance blood circulation time of NPs by providing a "stealth" effect, i.e., invisible to immune cell recognition (Owens and Peppas, 2006). Recently, a number of studies have been reported to characterize the role of the PEG conformation (i.e., brush or mushroom) and its impact on biomolecular corona formation.

The effect of PEG density on corona formation has been evaluated on numerous occasions. For example, NPs prepared from the particle replication in non-wetting templates (PRINT) method were prepared with two different PEG densities corresponding to the brush (0.083 PEG/nm^2) and mushroom (0.028 PEG/nm^2) regimes (Perry et al., 2012). Brush NPs displayed lower binding of bovine serum albumin (BSA) by nearly three-fold and four-fold less than non-PEGylated NPs. Significant differences between NPs with the two PEG conformations in terms of diminished macrophage uptake or increased circulation half-lives were not directly measured, but brush NPs performed better than mushroom NPs on average. At constant size, a similar result was obtained using AuNPs, where an increase in PEG grafting densities resulted in decreased serum protein adsorption (Walkey et al., 2011). In contrast, distinct differences were observed in terms of protein adsorption when size was considered. The same study found an inverse correlation between particle size and protein adsorption. The increased protein binding onto the smaller NPs was attributed to higher surface curvature and lower PEG-PEG steric interactions, which allowed a greater amount of the bare surface of the AuNP exposed (**Figure 1B**) (Walkey et al., 2011). When macrophage uptake was considered, two trends were observed. First, increased PEG density on similarly sized NPs resulted in decreased uptake. Second, at similar PEG densities, smaller NPs were taken up to a lesser extent than larger ones. Contrary to those results, in a study using PEGylated single-walled carbon nanotubes (SWCNT), brush SWCNTs were found to display shortened blood circulation times, faster renal clearance, and increased spleen vs. liver uptake, compared to mushroom SWCNTs (Sacchetti et al., 2013). Although these studies presented contrasting results with regard to PEG conformation, it is clear that the presence of PEG minimized biomolecular corona formation that was translated to enhanced pharmacokinetics of various NPs. However, to distinctly determine the role of PEG and PEG density in NP formulations, it is necessary to verify the biological properties of NPs in a case-by-case manner to obtain the desired response.

CONFORMATIONAL CHANGES OF ADSORBED PROTEINS CAUSED BY NPs

Achieving control over the toxicity of NPs is critical to ensure their optimal therapeutic effects. When a NP enters the body, it can alter the proteins that form its protein corona, and therefore induce toxicity during therapy. Some of these changes include alterations in protein conformation, protein function, and defective transport leading to the overexpression of inflammatory factors (Baugh and Donnelly, 2003; Wolfram et al., 2014a).

Many physicochemical properties of NPs affect protein adsorption, which influences how NPs interact with cells and tissues. The proteins adsorbed on the surface of the NPs can still be recognized as the native proteins by an interacting cell, and as a result, these denatured or misfolded proteins can trigger inappropriate cellular processes (Lynch et al., 2006). In a study investigating protein stability using silica NPs, conformational changes in protein variants of carbonic anhydrase II on NP surfaces occurred in a step-wise manner, where the least stable variants exhibited the quickest misfolding kinetics (Karlsson et al., 2000). When exposed to NPs for longer periods of time, all variants eventually folded into the same unstable state.

A number of studies have been dedicated to characterizing the interaction of albumin with NPs. AuNPs, for example, modified albumin from its stable secondary conformation to its unstable tertiary conformation (Shang et al., 2007). In the case of charged-PSt NPs, it was observed that albumin maintained its native secondary structure while associated with carboxylated NPs enabling its interaction with the albumin receptor. However, amine-terminated NPs denatured albumin and subsequently led to a loss of specificity toward the albumin receptor in favor for scavenger receptors, indicating that the transition to unstable proteins alters their activity in the body (Fleischer and Payne, 2014). This illustrated that the misfolding of proteins can result in an alteration of the cell surface receptors targeted by NPs, which could decrease their targeting efficacy (Fleischer and Payne, 2014).

Mortimer et al., investigated the role of scavenger receptors in NP-protein interactions (Mortimer et al., 2014). Albumin binding to synthetic layered silicate NPs (LSNs) induced protein unfolding akin to heat denaturation of albumin. Class A scavenger receptors, which are the dominant receptors involved in the mononuclear phagocyte system (MPS), required the presence of the albumin corona to recognize the LSNs.

The conformational changes of albumin can not only lead to increased NP clearance, but also alter their cellular uptake. A study characterized the biomolecular corona of negatively charged disulfide-stabilized poly (methacrylic acid) nanoporous polymer particles (PMA_{SH} NPPs) following incubation in complete media containing 10% fetal bovine serum (FBS). Adsorption of BSA, a major component in FBS, onto the surface of the NPPs was found to result in a conformational change from its native state. Notably, denatured BSA on NPPs caused a reduction in the internalization efficiency of the NPs into human monocytic cells, compared to the bare particles, due to reduced cell membrane adhesion. However, a different conformation of BSA, triggered class A scavenger receptor-mediated phagocytosis in differentiated macrophage-like cells (dTHP-1) without a significant impact on the overall degree of cell internalization. Recognizing that both composition and orientation of the protein corona are important for the assessment of biological interactions may lead to the prevention of off target cellular interactions of NPs (Yan et al., 2013).

CONSIDERATIONS OF THE BIOMOLECULAR CORONA FOR NP-BASED TARGETED DRUG DELIVERY

NP interactions with biomolecules can significantly affect the efficacies of nanomedicine. Alterations in conformations or activities of biomolecules can dramatically impair NP-based drug delivery. These alterations may result in changes in cellular uptake, drug release, and biodistribution profiles. Importantly, new methods to study those NP-cell interactions at the molecular level will yield insight into how the biomolecule corona can alter the fate of NPs (Bertoli et al., 2014).

In **Table 1**, we have summarized the major considerations one must take when designing and evaluating targeted NP drug delivery systems to achieve optimum efficacy.

Size is an important property of NPs that affects their distribution within the body. Biomolecular corona formation can increase the original size and alter the pharmacokinetics of NPs (Lundqvist et al., 2008). In some cases, this size increase could be beneficial since NPs smaller than 5 nm are readily excreted through renal filtration (Choi et al., 2007; Sunoqrot et al., 2014). Yet, the size increase caused by biomolecule adsorption may result in a

Table 1 | Considerations of the biomolecular corona to design more effective NPs.

		References
PHYSICOCHEMICAL PROPERTIES OF NPs AND THEIR EFFECT ON BIOMOLECULAR CORONA FORMATION		
Size	Larger NPs adsorb more proteins to their surfaces	Shannahan et al., 2013
Surface charge	Charged NPs adsorb more proteins to their surfaces. Alteration of particle zeta potential	Lundqvist et al., 2008
Hydrophobicity	More hydrophobic NPs adsorb more proteins to their surfaces.	Cedervall et al., 2007; Lindman et al., 2007
IMPACT OF PEG LAYERS ON BIOMOLECULAR CORONA FORMATION		
High density brush PEG conformation adsorbed less protein than mushroom conformations		Walkey et al., 2011; Perry et al., 2012
CONFORMATIONAL CHANGES OF ADSORBED PROTEINS CAUSED BY NPs		
Results in protein misfolding (changes in secondary structure)		Karlsson et al., 2000; Shang et al., 2007; Fleischer and Payne, 2014
Cryptic epitope exposure upon protein interaction with NP		Mortimer et al., 2014
Inappropriate receptor recognition after NP-protein interaction		Yan et al., 2013
CONSIDERATIONS OF THE BIOMOLECULAR CORONA FOR NP-BASED TARGETED DRUG DELIVERY		
In vitro experiments should be carried out in physiologically relevant conditions		Mirshafiee et al., 2013; Salvati et al., 2013
PEG backfilling may be used to overcome the negative effects of the protein corona on targeted drug delivery systems		Dai et al., 2014
Biomolecular corona formation can enhance pharmacokinetics of NPs		Peng et al., 2013
Biomolecular corona formation mitigates the toxicity of NPs		Ge et al., 2011; Mortensen et al., 2013

decreased therapeutic efficacy of NPs for diseases such as pancreatic cancer that require nanotherapies with particles sizes smaller than 50 nm (Cabral et al., 2011).

Considering those changes caused by the biomolecular corona, it appears essential to characterize the therapeutic and targeting efficacies of NPs under relevant conditions. Silicon dioxide (SiO_2) NPs were functionalized with Tf to validate their ability to maintain targeted interactions in physiologically relevant cell culture conditions. In FBS-containing medium, Tf-functionalized NPs lost their ability to selectively target A549 lung cancer cells (**Figures 1C,D**) (Salvati et al., 2013). Mirshafiee et al., prepared 75 nm SiO_2 NPs and studied their ability to react with synthetic, surface-bound azide groups using copper-free click chemistry (Mirshafiee et al., 2013). The results of this study confirmed that the biomolecular corona creates a barrier that screens the interaction of the ligand and its target on a separate surface.

While NP characteristics, such as size, shape, and surface charge, change due to biomolecular corona formation, drug release kinetics from the NPs can either be enhanced or disrupted. Liposomes can undergo shrinkage due to osmotic forces and may undergo a burst-release effect upon entering the blood, resulting in rapid drug release (Wolfram et al., 2014b). In contrast, protein binding on NPs has been shown to delay drug release, which prevented drug diffusion through the NP matrix (Paula et al., 2013) and reduced the burst effect (Behzadi et al., 2014).

The biomolecular corona may alter the toxicity profiles of NPs in a positive manner as well. Evidence has accumulated that the biomolecular corona may mitigate NP-induced toxicities. Decreased negative cellular impacts of carbon nanotubes were observed when they were coated with plasma proteins. Nanotubes with a higher protein density displayed less toxicity than those with a lower protein density (Ge et al., 2011). The effect of the biomolecular corona of 22 nm silica NPs with different surface charges on toxicity was also evaluated. The corona formed on each NP was confirmed to be unique, and SiO_2-COOH NPs exhibited lower toxicity than bare SiO_2 and SiO_2-NH_2 (Mortensen et al., 2013). These results indicated that NP-protein interactions can be utilized to reduce toxicities of some NPs that are otherwise known to be toxic to biological systems.

CONCLUSIONS AND FUTURE DIRECTIONS

The biomolecular corona has been demonstrated to have a major impact on the biological behaviors of NPs. Physicochemical properties of NPs including size, surface charge, and hydrophobicity affect the relative amounts, types, and conformations of proteins that adsorb onto the NP.

NPs functionalized with disease-specific targeting ligands are positioned to revolutionize the treatment of debilitating diseases such as cancer by achieving targeted and selective cellular interactions. However, the biomolecular corona diminishes those cellular interactions by making the ligands inaccessible at their surfaces. Therefore, development of strategies to overcome the negative impact of the protein corona on NP targeting is necessary. Recently, attaching targeting ligands to longer PEG tethers in combination with backfilling of the remaining bare surface with short PEG chains has been shown to promote the formation of targeted interactions *in vitro* (Dai et al., 2014). It is seemingly obvious that characterization and biological evaluations NPs must be performed in the presence of physiologically relevant protein levels, which will ultimately result in the enhanced *in vivo* efficacy of targeted drug delivery platforms.

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The application of magnetic nanoparticles for the treatment of brain tumors

Keon Mahmoudi[1] and Costas G. Hadjipanayis[2]*

[1] *Georgia Institute of Technology, School of Biology, Atlanta, GA, USA*
[2] *Brain Tumor Nanotechnology Laboratory, Department of Neurosurgery, Winship Cancer Institute of Emory University, Emory University School of Medicine, Atlanta, GA, USA*
**Correspondence: chadjip@emory.edu*

Edited by:
Jesús M. De La Fuente, Universidad de Zaragoza, Spain

Reviewed by:
Manuel Ocana, Consejo Superior de Investigaciones Científicas, Spain
María Luisa García-Martín, Andalusian Centre for Nanomedicine and Biotechnology, Spain

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INTRODUCTION

Glioblastoma (GBM), a World Health Organization (WHO) grade IV astrocytoma, is the most common and difficult primary brain tumor to treat (Braun et al., 2012). Even when detected early, the median survival rate for patients is 12–15 months (Adamson et al., 2009; Johnson and O'Neill, 2012). The challenge in treating GBM arises from its resistance to therapies such as radiotherapy and chemotherapy. GBM tumors are quite infiltrative into the surrounding normal brain permitting tumors to recur locally in the majority of patients.

The current standard of care treatment for GBM involves surgery and radiation, with concurrent and adjuvant chemotherapy (Stupp et al., 2005). Surgery permits the bulk of a GBM tumor to be removed in most cases. All patients have residual tumor cells residing away from the resection cavity that eventually lead to local tumor recurrence and the demise of the majority of patients (Hou et al., 2006). The infiltrating GBM cells reside centimeters away from the main tumor mass in normal brain making it difficult for complete surgical removal (Kim et al., 2014). Chemotherapy and radiotherapy of patients after surgery attempts to target these cells to prolong overall patient survival. The blood brain barrier (BBB) represents another challenge to the treatment of GBM tumors by preventing the accumulation of most chemotherapeutics into the brain to target the infiltrative cancer cells (Salazar et al., 1976; Bidros and Vogelbaum, 2009). Surgery and adjuvant therapies pose risks to the patient such as neurologic deficits and systemic toxicities. Known side effects of radiation therapy with chemotherapy for brain tumors include chronic fatigue, nausea, and cognitive deficits (Loehrer et al., 2011).

The BBB remains a formidable challenge in the treatment of GBM and malignant brain tumors. Its selective permeability is due to the presence of specialized endothelial cells, astrocytes, pericytes, and neuronal terminals (Tajes et al., 2014). The semi-permeable membrane that comprises the BBB prevents sufficient exposure of tumors to most chemotherapeutic drugs that are commonly used to fight tumor progression (Liu et al., 2010). Local disruption of the BBB is found within GBM tumors. The tumor vessels in GBM tumors are abnormal both structurally and functionally (Batchelor et al., 2007). The abnormal tumor vessels further impair delivery of therapeutics and create a hypoxic microenvironment that can reduce the effectiveness of radiation and chemotherapy. Antiangiogenic therapy attempts to normalize the tumor vasculature and improve the tumor microenvironment (Jain, 2001, 2005). Outside of the main tumor mass, the BBB is intact where brain cancer cells infiltrate into the surrounding normal brain. The oral chemotherapy agent, temozolomide (Temodar), can penetrate the BBB and has resulted in prolongation of overall survival patient survival by several months (Stupp et al., 2005).

The challenges associated with the treatment of GBM tumors require novel approaches for a greater impact on patient survival and quality of life for patients.

MAGNETIC NANOPARTICLES (MNPs)

MNPs are most commonly comprised of ferromagnetic iron-oxide (Fe_3O_4). They are invisible to the naked eye, typically measuring 1–100 nm in diameter (Sandhiya et al., 2009). MNPs can be designed to target cancer by modification of their surface with the addition of a peptide or antibody specific to cancer cells (Hadjipanayis et al., 2010). For biomedical applications, they can deliver targeted therapy to specific regions of the body. MNPs can be administered into the blood stream systemically and directed to a target with application of an external magnetic field (Pankhurst et al., 2003). Particles can be engineered to carry a drug, which can be released once the particles reach their target. *In vivo* experiments have shown the effects of MNPs within a magnetic field on glioma cells lasting up to 100 min postexposure (Braun et al., 2012). In a separate study with rabbits, intravenous injection of specially designed MNPs and subsequent exposure to an external magnetic field resulted in permanent remission of squamous cell carcinoma tumors (Chertok et al., 2008). While intravenous administration is feasible with tumors in other parts of the body, the BBB remains a formidable challenge for systemic delivery of agents for treatment of brain tumors. For the treatment of patients with GBMs, direct intratumoral delivery provides the

greatest concentration of therapeutic while minimizing systemic toxicities.

MRI CONTRAST ENHANCEMENT OF BRAIN TUMORS

MNPs also serve as a powerful aid for the imaging of brain tumors. Their inherent ferromagnetic qualities provide sensitive contrast enhancement with MR imaging (Liu et al., 2010). Accumulation of MNPs in brain tumors appears as a hypointensity on T2-weighted imaging including gradient echo imaging (Na et al., 2007). Functionalized MNPs can be engineered to target brain cancer cells which can in turn be identified with MR imaging. For standard visualization of tumors, MNPs can provide more sensitive imaging of tumors when used as a contrast agent for MRI (Kumar et al., 2010).

Ultrasmall superparamagnetic iron oxide nanoparticles (USPIONPs), a subclass of superparamagnetic MNPs, are the most effective types of MNPs that can be used for imaging purposes (Thorek et al., 2006). Their systemic half-life is two to three times greater than standard MNPs and are capable of being imaged by MRI for longer periods of time (Varallyay et al., 2002). In a recent study, it was noted that USPIONPs can be used to detect areas within brain tumors with increased blood flow, which may be indicative of tumor recurrence (Gambarota and Leenders, 2011). They can also be used to identify areas of pseudoprogression in brain tumors after standard adjuvant therapies such as radiotherapy and chemotherapy (Gahramanov et al., 2011).

HYPERTHERMIA

Hyperthermia for the treatment of different cancers has been well described in the past. Elevation of targeted areas of the body above 40°C can result in cancer cell death (Wust et al., 2002). In one study, researchers concluded that even moderate hyperthermia at a temperature around 45°C was enough to cause tumor cells to undergo apoptosis (Pu et al., 2013). Furthermore, local or regional hyperthermia can result in elevated blood flow, which may assist in the delivery of other treatments, such as chemotherapy, which could result in a synergistic antitumor effect (Kampinga, 2006; Issels, 2008).

While local or regional hyperthermia can be effective in treating cancer involving different parts of the body, treating brain tumors is difficult due to the surrounding skull (Jordan et al., 1999). Heat applied to the head is shielded by the skull which results in less than optimal temperature increases in the brain. Temperature elevation of the entire brain for prolonged periods of time would result in side effects and toxicities to patients. To provide a more targeted hyperthermia effect for brain tumors, MNPs may be delivered intratumorally prior to treatment with alternating magnetic fields. This process, known as thermotherapy, aims to deliver a greater hyperthermia effect locally to brain tumors while minimizing heating of the surrounding brain.

THERMOTHERAPY

Due to the side effects and toxicities of subjecting the entire brain to hyperthermia for extended periods of time, localized treatment is necessary for effective brain tumor therapy. Direct implantation of MNPs into brain tumors can bypass the BBB and allow for a maximum hyperthermic effect provided in a targeted manner **Figure 1**. Brain autopsies of two GBM patients after MNP injection, confirmed that the MNPs were retained within tumor tissue after implantation (Van Landeghem et al., 2009). Once injected into tumors, MNPs are subjected to an alternating magnetic field (AMF) which produces heat via the Brownian Néel relaxation process (Thiesen and Jordan, 2008; Deissler et al., 2014).

The localized hyperthermic effect, known as thermotherapy, involves the application of an alternating magnetic field (Maier-Hauff et al., 2011) **Figure 1**. When applying a magnetic field to the target area, the strength of the hyperthermic treatment is dependent on a variety of factors including the strength of the AMF, the size and concentration of the MNPs, and the time in which the field is applied to the tumor region (Yanase et al., 1997; Guedes et al., 2004; Meenach et al., 2010). Targeted treatment is necessary because prolonged application of hyperthermia to healthy tissue can result in unwanted side effects and toxicities (Fajardo, 1984). In order to minimize the risk of systemic toxicities, the hyperthermic treatment is only applied for a brief period of time to allow for the MNPs within the targeted region to heat up and cause necrosis or death of the cancer cells. In human patients with brain tumors, it was determined that hyperthermia with temperatures from 42°C to 49°C were safe and caused very few side effects for the patient (Maier-Hauff et al., 2007).

Thermotherapy does induce the death of malignant cells (Marcos-Campos et al., 2011). When a MNP is subjected to an alternating magnetic field, its internal temperature increases. This heat is then transferred locally to the abnormal cells situated around the nanoparticles which further results in tumor death (Fajardo, 1984). With thermotherapy, only the targeted tumor region is exposed to increased temperatures, resulting in localized necrosis. When clinicians studied the benefits of using thermotherapy in conjunction with radiotherapy in relapsed GBM, they reported an overall survival of 13.4 months compared to just 6.2 months with radiotherapy and Temozolomide alone (Maier-Hauff et al., 2011). Current limitations to the use of MNPs for thermotherapy of brain tumor patients include the high MNP concentration required to generate hyperthermia precluding the use of MRI, as well as the effective delivery of the MNPs (Wankhede et al., 2012).

The decreased resistance to heat observed in GBM cells is not as clearly presented when conducting experiments with *in vitro* samples (Issels, 2008). Cancer cells that reside in tumors are more susceptible to damage from heat than cancer cells that are *in vitro* (Rhee et al., 1990). This contrasts heavily with the significant difference in immunity that is observed when experiments are conducted using *in vivo* models. One explanation for this difference is that the vascular network within the tumor is abnormal which can lead to areas that have a difference in pH as well as decreased availability of oxygen (Issels, 2008).

CONCLUSION

Thermotherapy involving the use of an AMF in conjunction with MNPs has proven to be an effective method for treating patients with GBM. Initial tests have shown that MNPs have minimal toxicities to patients, though further testing must be done to confirm these findings

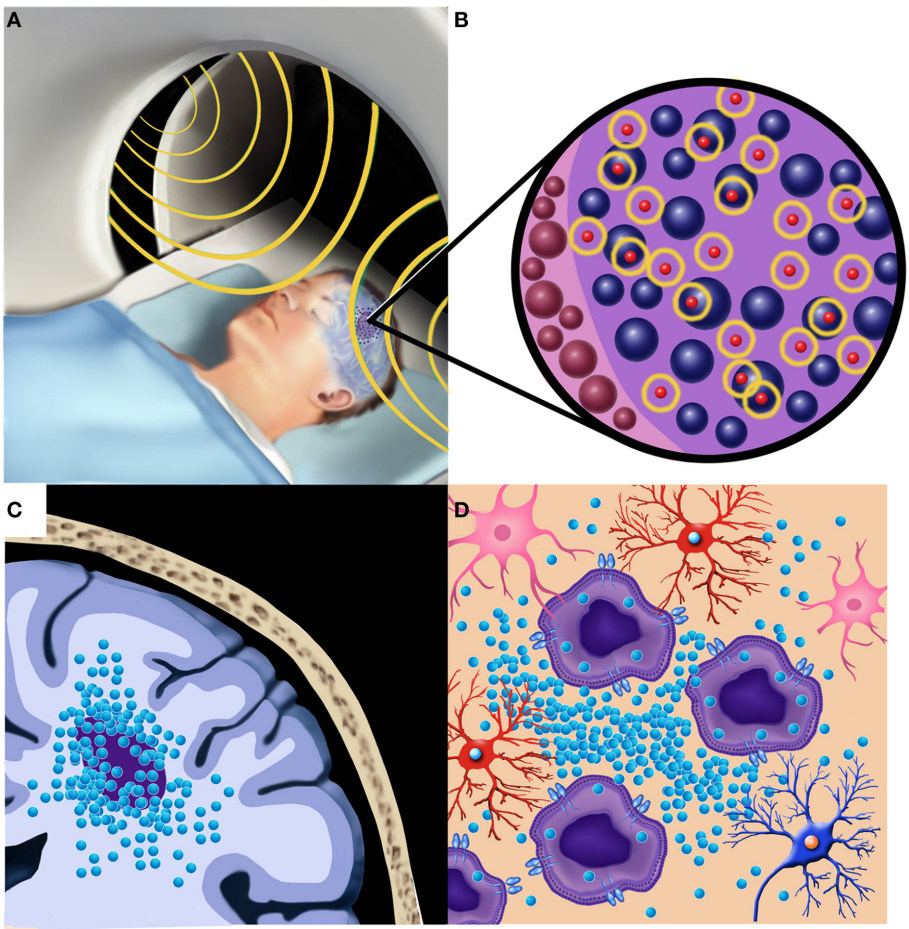

FIGURE 1 | Local hyperthermia treatment of a patient with a malignant brain tumor after implantation of MNPs. (A) The patient undergoes an alternating magnetic field (AMF) session (shown in yellow) for generation of local hyperthermia in the region of the tumor. **(B)** Oscillation of the MNPs (shown in yellow) within and adjacent to the tumor cells provides the therapeutic hyperthermia (thermotherapy) effect. **(C)** Local implantation of the MNPs within and adjacent to the brain tumor provides a targeted therapeutic effect. **(D)** Brain tumor cells shown infiltrating normal brain may be more susceptible to the effects of local hyperthermia by greater MNP intracellular uptake and sensitivity to temperature changes.

(Mahmoudi et al., 2012). Much like other methods that are used to combat GBM, MNPs do not serve as a cure on their own; they have shown to be most effective when used as an adjuvant therapy with other treatment modalities. Combining fractionated radiotherapy with thermotherapy has shown a survival advantage in patients with relapsed GBM (Maier-Hauff et al., 2011).

With further research, scientists can bioengineer multitasking MNPs that can be used for imaging, drug delivery, and localized thermotherapy (Hadjipanayis et al., 2013). Better targeting of MNPs may provide more effective treatment of GBM. The bioconjugation of drugs, monoclonal antibodies, or peptides specific to cancer cells will improve targeting. MNPs appear to be well tolerated when delivered directly into the human brain with few side effects associated with them. Further testing of MNPs with standard of care chemotherapy, such as temozolomide, needs to be completed in patients with malignant brain tumors. MNPs will likely assume a larger role in brain cancer treatment, with other adjuvant therapies being used to complement magnetic nanoparticles (Kim et al., 2014).

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Molecular imaging of breast cancer: present and future directions

David Alcantara*, Manuel Pernia Leal, Irene García-Bocanegra and Maria L. García-Martín

Laboratory of Metabolomics and Molecular Imaging, BIONAND, Centro Andaluz de Nanomedicina y Biotecnología (Junta de Andalucía, Universidad de Málaga), Malaga, Spain

Edited by:
João Conde, Massachusetts Institute of Technology, USA

Reviewed by:
Marc Poirot, Institut National de la Santé et de la Recherche Médicale, France
Giuseppe Esposito, Georgetown University Hospital, USA

****Correspondence:***
David Alcantara, Laboratory of Metabolomics and Molecular Imaging, Centro Andaluz de Nanomedicina y Biotecnologia (BIONAND), C/Servero Ochoa, 35, 29590 Campanillas, Malaga, Spain
e-mail: dalcantara@bionand.es

Medical imaging technologies have undergone explosive growth over the past few decades and now play a central role in clinical oncology. But the truly transformative power of imaging in the clinical management of cancer patients lies ahead. Today, imaging is at a crossroads, with molecularly targeted imaging agents expected to broadly expand the capabilities of conventional anatomical imaging methods. Molecular imaging will allow clinicians to not only see where a tumor is located in the body, but also to visualize the expression and activity of specific molecules (e.g., proteases and protein kinases) and biological processes (e.g., apoptosis, angiogenesis, and metastasis) that influence tumor behavior and/or response to therapy. Breast cancer, the most common cancer among women and a research area where our group is actively involved, is a very heterogeneous disease with diverse patterns of development and response to treatment. Hence, molecular imaging is expected to have a major impact on this type of cancer, leading to important improvements in diagnosis, individualized treatment, and drug development, as well as our understanding of how breast cancer arises.

Keywords: breast cancer, molecular imaging of breast, breast cancer diagnosis, contrast agents, breast imaging techniques, breast magnetic resonance imaging

INTRODUCTION

Modern clinical cancer treatments require precise positional information. Where is the tumor located? How large is it? Is it confined, or has it spread to the lymph nodes? Does it involve any critical anatomical structures that would alter the treatment strategy? These questions are being answered, at ever-increasing spatial resolution, through the application of traditional anatomical imaging methods such as computed x-ray tomography (CT), magnetic resonance imaging (MRI), and ultrasound (US). Although these methods still represent the mainstay of clinical imaging, it has become clear that the acquisition of molecular and physiological information by nuclear magnetic resonance and optical imaging technologies could vastly enhance our ability to fight cancer (Weissleder, 2006).

Emerging genomic and proteomic technologies have the potential to transform the way in which breast cancer is clinically managed. Molecular imaging is poised to play a central role in this transformation, because it will allow the integration of molecular and physiological information specific to each patient with anatomical information obtained by conventional imaging methods. The hope is that clinical molecular imaging will one day be used to achieve the following: (i) the early detection of molecular or physiological alterations that signal the presence of cancer when it is still at a curable stage, (ii) the ability to evaluate and adjust treatment protocols in real time, and (iii) the ability to streamline the cancer drug development process.

The development of new breast cancer therapeutics is expensive, time-consuming, and often requires vast numbers of patients. Molecular imaging is currently one of the most powerful non-invasive techniques used in clinical diagnosis that exhibits a high potential to improve the efficiency and cost-effectiveness of drug development programs. In this article we present a short review on the main techniques and the perspectives of future Breast Cancer Imaging.

IMAGING TECHNIQUES FOR BREAST CANCER

Mammography and ultrasound are the most common methods used for diagnosis and guided intervention in breast disease. The relevance of breast MRI has been also increased, fulfiling an important role in operated breasts and suspicious lesions. Multiple diagnostic techniques, including tomosynthesis, mammography and ultrasound contrast elastography, 3D ultrasound, diffusion and perfusion and breast spectroscopy, have also been developed. Moreover, the use of the American College of Radiology (ARC) BIRADS scale (Breast Imaging Reporting and Data System) has been implemented in diagnostic centers during the last decade (American College of Radiology, 2003). BIRADS classification started in the late 1980s to address a lack of standardization and uniformity in mammography practice reporting (McLelland et al., 1991). The BIRADS lexicon provided new opportunities for quality assurance, communication, research, and improved patient care. Many well-respected groups participated in this development initiative to establish a broad base of support (D'orsi and Kopans, 1997; Burnside et al., 2009; Mercado, 2014).

The BIRADS scale is a classification of breast disease according to radiological findings that includes six grades of malignancy and indicates the actions that must be followed for each grade (**Figure 1**). The implementation of BIRADS has allowed us to

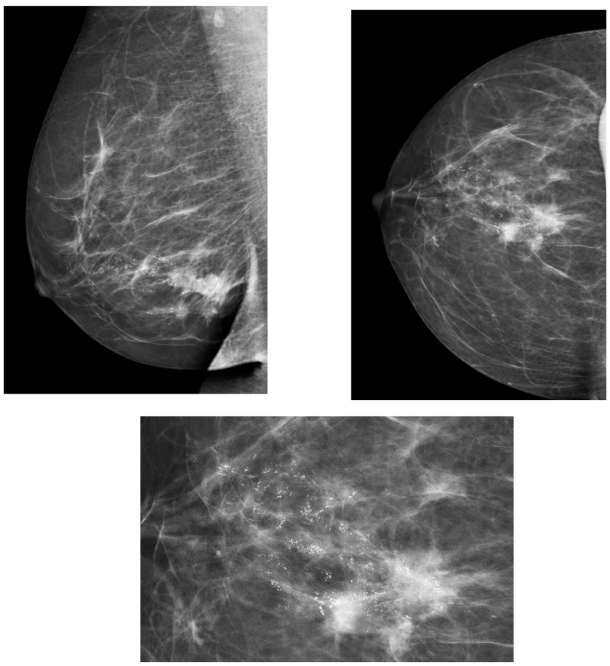

FIGURE 1 | Microcalcifications in breast mammography. Highly suggestive of malignancy BIRADS 5.

homogenize the diagnosis and injury treatment methods in all centers.

BIRADS CLASSIFICATION

Category 0: Additional imaging evaluation and/or comparison to prior mammograms is needed.
Category 1: Negative.
Category 2: Benign (non-cancerous) finding.
Category 3: Probably benign finding – Follow-up in a short time frame is suggested.
Category 4: Suspicious abnormality – Biopsy should be considered:
Category 4A: Finding with a low suspicion of being cancer.
Category 4B: Finding with an intermediate suspicion of being cancer.
Category 4C: Finding of moderate concern of being cancer, but not as high as Category 5.
Category 5: Highly suggestive of malignancy – Appropriate action should be taken.
Category 6: Known biopsy-proven malignancy – Appropriate action should be taken.

The main diagnosis and monitoring techniques of breast disease include mammography, ultrasound and magnetic resonance imaging (MRI).

MAMMOGRAPHY

Mammography is the most commonly used method for the monitoring and diagnosis of breast disease. In recent years, digital mammography has been developed, which requires lower doses of radiation as compared to a conventional mammography, and also allows the post-processing of images.

Mammography is also used to guide interventional breast techniques, such as stereotactic mammography. Two mammographic views, cranio-caudal and oblique medium lateral are usually performed and complemented with other projections according to needs. This technique has high sensitivity for the diagnosis of microcalcifications. It is also used for breast screening, allowing the detection of breast lesions at a very early stage, which increases considerably the life expectancy of affected patients. According to the screening program, a mammography is usually performed every two years (two projections) in women over the age of 40 or 50, with double read by two different radiologists (Houssami et al., 2009).

However, mammography still present some drawbacks. Firstly, it is an ionizing technique, and although the radiation dose has considerably decreased, it is still relevant if we take into account that the breast is a radiosensitive tissue. Secondly, mammography cannot differentiate between liquid lesions, including cysts, and solid lesions, which is a major limitation for the accurate identification of tumor masses.

Two innovative techniques are included within mammography:

Contrast-enhanced mammography

Contrast mammography, as well as MRI, allows dynamic vascular studies to be performed. Several parameters can be extracted from the enhancement curve that provide useful diagnostic information, such as the slope and the time to peak. Thus, lesions with early and intense enhancement are suggestive of malignancy (Fallenberg et al., 2014).

This type of study has been satisfactorily used for the analysis of inconclusive lesions, the detection of occult lesions, the monitoring of disease progression and to assess chemotherapy response (Dromain et al., 2009).

Tomosynthesis

Tomosynthesis techniques allow us to carry out three-dimensional breast studies. It consists of a mammography device that uses a rotary head tube, performing different projections of a static breast with a specified angle (between 15° and 45°). It can be considered a tomographic application of digital mammography. Tomosynthesis has even been proposed as a new screening method (Waldherr et al., 2013). Tomosynthesis has demonstrated superior accuracy compared to mammography in tumor measurements and reduced the suspicious presentations of normal tissues and tissue overlap, and facilitated accurate differentiation of lesion types (Fornvik et al., 2010; Alakhras et al., 2013).

ULTRASOUND

Ultrasound complements mammography, being a required method for the management and diagnosis of breast pathology (**Figure 2**). Because it does not use ionizing radiation, ultrasound is not only the first diagnostic tool in young women who have little risk of breast cancer, but also the first diagnostic technique in

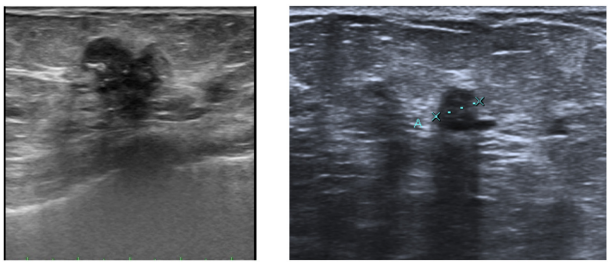

FIGURE 2 | Breast cancer ultrasound images.

pregnant and breastfeeding women. Moreover, it has high sensitivity to show tumor margins and the internal characteristics of tissue, and it is used as complementary technique to mammography for the study of dense breasts and to assess lymph node status. It is also frequently used for breast intervention, for guided biopsy and for the placement of harpoons. In recent years, different ultrasound techniques for the breast pathology studies have been developed, including:

Ultrasound contrast

Ultrasound contrast involves the intravenous injection of perfluorocarbon microbubbles to observe the behavior of breast lesions by ultrasound. The perfusion area and the signal intensity curves in relation to time are obtained using this method. Ultrasound contrast is used for diagnosis, detection of recurrence and monitoring of treatment response, and is particularly useful as a guide for puncture of suspicious lymph nodes, as metastatic lymph node areas do not capture contrast and can be therefore differentiated from healthy areas.

On the other hand, 3D ultrasound is particularly useful for the study of breast lesion with contrast, because it allows the assessment of nodes with contrast in three dimensions (Jia et al., 2014).

Elastography

This technique is based on the same principle as breast tenderness and can determine the hardness of the lesion by measuring the elastic properties of tissues by ultrasound, as the lower the hardness of a lesion, the higher the probability of being benign, and vice versa. Elastography has been shown to be very useful in the assessment of benign lesions (BIRADS 3) (Itoh et al., 2006; Scaperrotta et al., 2008).

MAGNETIC RESONANCE IMAGING (MRI)

MRI is an important diagnostic tool frequently used to study breast disease. It currently has specific indications, including evaluation of response to treatment, screening in high-risk patients, study of occult breast cancer, study of tumor recurrence and assessment of breast prostheses. MRI can be also recommended for the staging of breast cancer, the study of microcalcifications, breast discharge, premalignant lesions, residual tumor in operated patients or in case of inconclusive findings by mammography and ultrasound (Mann et al., 2008; Sardanelli et al., 2010).

The MRI techniques applied to the study of breast cancer are based on both the assessment of the morphological features of the lesions and the characteristics of contrast enhancement of these lesions. Malignant tumors have a disorganized angiogenesis showing specific morphological and functional characteristics.

The MRI study includes pre-contrast T2 sequences and post-contrast 3D T1-weighted gradient Eco. Several parameters are analyzed in the contrast studies, such as the slope of the enhancement curve during both the uptake and wash out phases, the time to peak enhancement or the maximal relative enhancement. These analyses can differentiate benign from malignant lesions. Image post-processing plays an important role in dynamic contrast MRI because it provides the radiologist with additional parametric information that can be crucial for a more accurate diagnosis. Thus, typical post-processing processes include subtraction of images, projections of maximum signal intensity (MIP), multiplanar reconstructions (MPR) and time curves of suspicious lesions uptake (Kuhl, 2007; Partridge, 2008).

Currently, there are two classification systems for the diagnostic criteria in breast MRI: the Fischer and the ACR classifications. Both present common diagnostic criteria, integrating morphological and dynamic uptake information. The ACR classification presents common criteria with the BIRADS classification of mammography and ultrasound (Agrawal et al., 2009; Morris Ea et al., 2013). During the last few years, other MR techniques have been proposed for the study of breast cancer, namely diffusion MRI and spectroscopy.

Diffusion MRI

Diffusion MRI techniques are based on the application of field gradients to enhance the signal lost due to the Brownian motion of water molecules. The diffusion weighting is determined by the strength and duration of the diffusion gradients, and the time between the gradient pulses, which is all quantified by the b-factor. The exponential fit of signal intensity vs. the b values provides the apparent diffusion coefficient (ADC), whose values reflect the restriction of water motion in any given tissue and, in the case of tumor growth, it has been related to cellularity. Malignant tumors usually have high cellularity and therefore present low ADC values as compared to benign lesions. Diffusion MRI is a quick technique that does not require the use of contrast agents and its implementation has been recommended as part of a routine protocol for breast MRI. The main disadvantages of diffusion MRI are the low spatial resolution and the lack of specificity to differentiate between benign and malignant tumors (Guo et al., 2002; Peters et al., 2008).

MR spectroscopy

Breast spectroscopy provides information about the metabolic profile of tumor tissue, being the most important metabolite in breast spectroscopy tCho (total choline), which has been related to tumor proliferation activity. The use of spectroscopy has been shown to increase the specificity of MR for the differentiation of benign and malignant lesions. Several studies indicate the association between the choline peak with the response to the treatment. In this sense, Tozaki et al. (2010) found that the reduction of choline peak is more sensitive to determining the response to

treatment than the decrease of tumor size. Furthermore, the combination of spectroscopy and diffusion MRI data have demonstrated a high specificity to characterize benign and malignant lesions (Tsougos et al., 2014).

CONTRAST AGENTS

In spite of the multiple image diagnostic tools commented on above, radiologists still find some difficulties when diagnosing early stage breast tumor malignancies due to the lack of sensibility given for these techniques. Early detection has become one of the most important issues in the cancer treatment, and researchers have improved this issue by developing external substances called contrast agents that enhance the sensibility of images in the region of interest. This fact allows improvement to the quality and the follow-up of molecular processes at the cellular and molecular levels of the region under study. The more common contrast agents used in clinical studies are gadolinium- and iodine-based structures for magnetic resonance imaging and mammography (X-ray) respectively, however other contrast agents such as radiotracers are being investigated as potential biomarkers for daily clinical practice.

MAGNETIC RESONANCE IMAGING CONTRAST AGENTS

As noted above, the gadolinium-based contrast agents are the most commonly used in clinical practice, generating a positive image of the nearby tissues. The presence of gadolinium ions shortens the T_1 relaxation time, thus generating an increase of the intensity in the images (bright signal or positive image) that help to distinguish malignancies from other benign pathologies (Zhou and Lu, 2013). However, gadolinium is a high toxic paramagnetic cation (Gd^{3+}) that needs to be protected from the body, so gadolinium is mainly reacted with chelate ligands thus minimizing the toxicity effects from the free gadolinium (Gd^{3+}). Therefore, the most typically used gadolinium chelates as MRI contrast agents in clinical practice are gadopentetate dimeglumine (Gd-DTPA, Magnevist®), gadoterate dimeglumine (Gd-DOTA, Dotarem®), gadoteridol (Gd(HP-DO3A), Prohance®), and gadodiamine (Gd(DTPA-BMA), Omniscan®). These contrast agents, approved by the Food and Drug Administration (FDA), present excellent biodistribution within the extracellular space, and fast renal clearance from the body with half-lives of 1–2 h. Albeit the most used gadolinium-based contrast agents are the Gd-chelates, complex gadolinium-based contrast agents have been developed to improve their T1 relaxation time and pharmacokinetics. These complexes provide well-defined advantages over Gd-chelates due to slow rotational motion, they are a combination of Gd-chelates, such as Gd-DTPA and Gd-DOTA, and dendrimers (Li et al., 2013) or liposomes (Huang and Tsourkas, 2013; Zhou and Lu, 2013). For instance, poly(amidoamine) PAMAM dendrimers in different generations have shown higher r1 relaxivity and size-dependent pharmacokinetics, low generation (2–4) presented renal clearance, and high generation (5–10) presented minimal renal clearance. A recent modification in the design of contrast agent dendrimers resulted in the development of dendrimer nanoclusters (DNCs), as a combination of small PAMAM dendrimers. These DNCs were easily synthesized in high yields and also exhibited higher r1 values than the small units of PAMAM-based contrast agents (Cheng et al., 2010). These Gd-chelates were also combined with liposomes via encapsulation in the inner core or immobilization at the liposome surface, being the immobilization most used since the relaxivity is higher than in the encapsulated method, thus exhibiting a low water exchange rate with the gadolinium encapsulated in the inner core of the liposome.

X-RAY CONTRAST AGENTS

Iodine-based contrast agents improve the visualization of images in radiography and CT by increasing the density of tissues and also the vessels. Similarly to dynamic MRI using Gd chelates, relevant information can be extracted from the different uptake kinetics of these contrast agents that helps in differentiating malignant lesions from benign tissues. In case of malignant tissues, the iodine is rapidly absorbed and desorbed (more contrast), while in the benign ones the absorption takes place slowly (less contrast), thus giving differences in the tissue density of the images. Iodine contrast agents could be sorted into two groups depending on the binding to the iodine: covalent (non-ionic) or ionic contrast agents (Robbins and Pozniak, 2010). The ionic contrast agents present are better contrast agents than the non-ionic ones due to their higher osmolality, with the consequently increased delivery or disassociation of iodine (Barrett et al., 1992). However, the ionic iodine contrast agents induces more toxicity (more side effects) mainly due to the large amount of iodine ions delivered (higher osmolality injected in comparison with serum), being also recently reported that these contrast agents could affect the thyroid in some patients (Rhee et al., 2012). Therefore, in order to minimize the toxicity, the contrast agents used for the clinical practice present values close to the serum, and non-ionic bonds such as Iohexol 300 mg Iodine/mL (Omnipaque) and Iodixanol 320 mg Iodine/mL (Visipaque).

POSITRON EMISSION MAMMOGRAPHY (PEM)

PEM is high-resolution PET scanner that provides functional imaging specifically for breast cancer detection (Kalles et al., 2013). PEM can isolate and enhance breast images with more accuracy than full-body PET scans and works much like a full-body PET scan (see Section PET and SPECT). In this technique, the contrast agents used in the evaluation of breast cancer are radiotracers (Penuelas et al., 2012), in particular radioactive labeled sugar-like molecules. These radiotracers help the diagnostic accuracy of the cancer, especially in the early stages, metastasis, and also cancer progression during the treatment. ^{18}F-fluorodeoxiglucose (FDG) (Caldarella et al., 2014) is the most typical radiotracer used as a contrast agent in PEM. FDG is an analog of glucose that is accumulated, after injection, mostly in cancer tissues, since those present a faster metabolism in comparison with the normal tissues allowing a more clear vision of the suspected malignant tissues by PEM. Another radiotracer is the 3′-deoxy-3′-[^{18}F]fluorothymidine (^{18}FLT). This radiotracer is not still used as a routine breast cancer contrast agent in clinical practice, but it has been tested as a biomarker for imaging cellular proliferation. ^{18}FLT is a structural analog of DNA nucleoside thymidine that is trapped in the cell thus informing about the stage or monitoring the evolution of the tumor cells (Caldarella

et al., 2014). In addition to the imaging of breast cancer proliferation and progression, the N-[^{11}C]methylcholine (^{11}C-choline) is used as a radiotracer (Contractor et al., 2009) since the choline is modified to phosphocholine due to the increase of the activity of the enzyme choline kinase-α as noted above in the MR spectroscopy section.

MOLECULAR IMAGING TECHNIQUES FOR BREAST CANCER

The term "molecular imaging" refers to the non-invasive visualization and measurement of biological processes at the cellular and molecular levels in a living system using endogenous or exogenous markers.

There are many different imaging modalities that can be used for molecular imaging, the most relevant ones being: nuclear imaging (PET and SPECT), optical imaging and magnetic resonance imaging.

The direct observation of endogenous markers can be achieved with magnetic resonance *in vivo* spectroscopic imaging (MRSI) (Begley et al., 2012; Bolan, 2013) or some advanced optical methods, such as Raman spectroscopy (Kallaway et al., 2013). The first one is based on classical nuclear magnetic resonance (NMR) spectroscopy, which allows the detection and quantification of molecules containing magnetic nuclei, typically ^{13}C, ^{31}P, ^{19}F or ^{1}H, being ^{1}H NMR the most widely used *in vivo*. The combination of NMR sequences with field gradients in MRI scanners allows for the spatial localization of the observable metabolites, giving rise to MRSI. Both ^{1}H and ^{31}P MRSI have been used for the metabolic characterization of breast tumors at a high magnetic field (Klomp et al., 2011). Raman spectroscopy is based on inelastic scattering of photons after interaction with vibrating molecules and thus provides information about tissue composition (Brozek-Pluska et al., 2012; Li et al., 2014).

The term molecular imaging, however, most commonly refers to the use of exogenous markers (contrast agents) to visualize and measure *in vivo* processes. For breast cancer diagnosis, PET and SPECT have been widely used in clinical practice, whereas MRI is expected to have a major impact in the near future, and optical imaging is mainly used in preclinical studies.

PET AND SPECT

PET imaging uses radioactive isotopes that emit positrons, *such as* ^{18}F, ^{15}O, ^{13}N, or ^{11}C; whereas SPECT imaging uses isotopes that emit gamma photons, such as ^{99m}Tc, ^{123}I, or ^{125}I. Positrons travel short distances in tissues, in the order of millimeters, and collide with surrounding electrons (annihilation), producing two high energy gamma rays that travel in opposite directions to one another and are detected by the PET camera. The time delay between the detection of paired opposite direction is used to calculate the location of the annihilation event. In SPECT, a single photon is emitted per event and detected by rotating gamma cameras.

Most PET radioisotopes are short-lived, ranging from a few minutes to 2 h, which implies the availability of an on-site cyclotron to produce them and therefore increases the cost of PET imaging dramatically. SPECT radioisotopes are longer-lived, in the order of hours (6 h for ^{99m}Tc), allowing for longer image acquisition times. On the other hand, PET shows higher sensitivity as compared to SPECT.

Both PET and SPECT provide information about physiological activity, such as glucose metabolism, blood flow and perfusion, and oxygen utilization (Kjaer, 2006). However, they lack anatomical detail, which has led to the development of hybrid systems that combine PET and SPECT with other image modalities, CT and MRI.

Both whole-body and dedicated PET/CT scanners are currently available. Dedicated systems have higher sensitivity allowing for the detection of small tumors and thus being more accurate for molecular imaging, whereas whole-body scanners provide valuable information for locoregional and distant staging (Koolen et al., 2012). PET/MRI is a more recent technology that offers the advantage of lower exposure to radiation and higher contrast resolution, together with the possibility of adding functional information from other MRI modalities, which has great potential for molecular imaging. However, further technological developments are still needed to get optimal performance of a fully integrated PET/MRI system (Pace et al., 2014). SPECT/CT has shown to be a valuable tool for sentinel lymph node detection (Husarik and Steinert, 2007; Lerman et al., 2007; Van Der Ploeg et al., 2009; Coffey and Hill, 2010).

OPTICAL IMAGING

Optical molecular imaging of the breast is based on the use of near-infrared (NIR) light to excite exogenous fluorescent probes that have been designed to selectively target breast tumor cells (Levi et al., 2007; Poellinger, 2012). The use of NIR-fluorophores for immunohistochemical characterization of excised tumor specimens is a common *in vitro* diagnostic technique. The goal of molecular imaging, however, is to detect these fluorophores *in vivo*, thus avoiding the need for biopsies. There are technical limitations, though, that need to be addressed if these methods are to be used on patients, like tissue penetration and background signal contamination. To date, the use of NIR optical imaging *in vivo* is limited to tumor xenografts in preclinical studies (Oliveira et al., 2012; Sano et al., 2012; Van De Ven et al., 2012) or intraoperative imaging for tumor margin detection and lymph node mapping (Lee et al., 2010; Verbeek et al., 2014).

MOLECULAR MAGNETIC RESONANCE IMAGING

MRI has attracted a great deal of interest in the era of molecular imaging, as it is the most versatile diagnostic imaging modality, able to provide excellent anatomical detail, together with functional and metabolic information (**Figure 3**). Furthermore, its non-ionizing nature offers the possibility of performing longitudinal follow-up studies without any risk for the patient.

The majority of the MRI signal comes from the water protons (^{1}H) and the contrast from the local differences in water content, water motion and magnetic relaxation times, T_1 and T_2, of the water protons. Although intrinsic contrast is sufficient for most MRI applications, the use of exogenous contrast agents is often required for accurate diagnosis. Most MRI contrast agents are based on either gadolinium chelates or superparamagnetic nanoparticles (SPIONs), these being the base for molecular imaging.

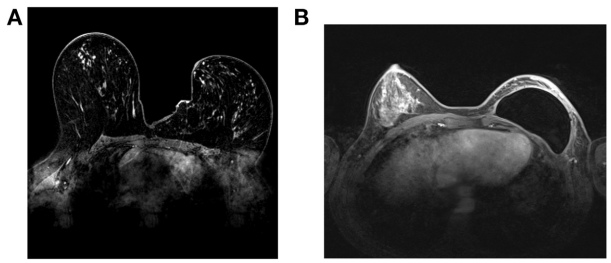

FIGURE 3 | Breast MRI images at 1.5T. A conventional breast MRI image (without contrast agent) **(A)**. A breast prosthesis can be seen in the right breast **(B)**.

In contrast to nuclear and optical imaging modalities, which are based on the direct detection of molecular probes, molecular magnetic resonance imaging is an indirect method that detects the effect of the contrast agents on the magnetic properties of the surrounding water molecules. This is a crucial aspect in understanding MRI-based molecular imaging, as explained below.

The major drawbacks of MRI for molecular imaging applications are, on the one hand, its inherent low sensitivity, due to the small difference in atoms between the high and the low energy states, and on the other hand, the lack of specificity of conventional MRI contrast agents. Thus, molecular imaging probes have to be able to strongly increase sensitivity and at the same time show high specificity. In this regard, SPIONs (Lodhia et al., 2010; Ittrich et al., 2013; Jin et al., 2014) have important advantages over other magnetic contrast agents because they produce signal enhancement through local field inhomogeneities, which affects the T2 of a large number of water molecules, thus leading to very strong signal enhancement. Nonetheless, other types of contrast agents have also been proposed for magnetic resonance molecular imaging, such as Gd-based nanoparticles (Huang and Tsourkas, 2013). Finally, the functionalization of these nanosystems using technological approaches adds both specificity and biocompatibility.

Some studies have already shown the great potential of magnetic resonance for molecular imaging of breast cancer using targeted nanoparticles (Li et al., 2013; Yan et al., 2013). Although these studies have been only conducted in animal models, it can be expected that, in the near future, the rapidly growing field of nanomedicine will facilitate the translation of these methodologies to the clinics.

PERSPECTIVES

Although molecular imaging is able to visualize breast tumor morphology and functional and metabolic processes within the tumor at several levels, the sensitivity of the different molecular imaging techniques is varied depending on the type of marker used in signaling the biological processes. At present, the main milestones for future molecular imaging development in breast cancer are:

1. To enhance knowledge of molecular drivers behind breast cancer subtypes, progression and metastasis.
2. To develop validated markers for chemosensitivity and radiosensitivity.
3. To validate multimodality imaging biomarkers for minimally invasive diagnosis and monitoring of responses in primary and metastatic disease.
4. To develop interventions and support to improve the survivorship experience.

In 2012, the charity Breast Cancer Campaign facilitated a series of workshops where specialists and other stakeholders revealed the main gaps in the prevention and treatment of breast cancer (Eccles et al., 2013). Top problems in molecular imaging of breast cancer (and recent research on the field) to be highlighted are:

1. There is a need to increase the use of functional screening techniques to learn about tumor heterogeneity, identify features associated with response or resistance to treatment and accelerate the rate at which promising ones enter clinical evaluation. The "Europe Against Cancer" programme has created quality assurance guidelines used for all mammography-based screening for breast cancer. They were created to maximize results while minimizing negative effects. The Mammography Quality Standards Act (MQSA) in the United States has mandated that all mammography clinics be certified (Von Karsa and Arrossi, 2013). Resistance to chemotherapy has brought to light the issue of tumor heterogeneity. Approximately 70% of human breast tumors are ER positive and depend on estrogen for growth. The use of selective ER modulators, such as tamoxifen, in ER-expressing tumors was one of the first examples for successful targeted therapy based on the tumor's molecular classification (Swaby et al., 2007). What induces endocrine resistance in these tumors has been one of the longest standing and most intense areas of breast cancer research. The somatic evolution of tumor progression was discovered in 2012, but the results raised additional questions that could not be answered at that time (Greaves and Maley, 2012). One of the most exciting outcomes of comprehensive cancer-genome-sequencing studies is that we finally have the tools to follow clonal and subclonal evolution of tumors and see the complexity of cancers as a whole (Polyak, 2014).
2. Evaluation of emerging imaging biomarkers of primary and metastatic breast cancer. A biomarker is a crucial tool for measuring the progress of disease and the effects of treatment for better clinical outcomes in breast cancer patients. The current questions of therapeutic choices can focus now on the understanding that breast cancer is truly a collection of genetically-specific heterogeneous diseases, each demonstrating different clinical behavior and therapeutic response (De Mattos-Arruda et al., 2013). Several biomarkers have been proposed as new breast cancer targets, including MicroRNAs (mi-RNA) (Mulrane et al., 2014), proteins (Kondo, 2014), antibodies (Knowles and Wu, 2012), or glycans (Adamczyk et al., 2012). One promising direction is the detection and imaging of circulating cell-free DNA (cf-DNA). Since 2002, cf-DNA has been shown to represent a good non-invasive biomarker, as it can be isolated from human plasma, serum

and other body fluids (Utting et al., 2002). It was also reported that the concentration of DNA in the bloodstream of patients with breast cancer was higher than healthy controls (Fleischhacker and Schmidt, 2007). Thus, the detection of cf-DNA provides new opportunities for management of cancer patients, adding a useful new tool for diagnosis, staging and prognosis (Esposito et al., 2014). Imaging of cf-DNA after chemotherapy treatment has been described by using fluorochrome-functionalized nanoparticles (Cho et al., 2013). Very recently, cf-DNA from plasma samples has been imaged by AFM and allowed to confirm the specific size pattern of tumor-derived cf-DNA (Mouliere et al., 2014).

3. Increased specificity and improved clinical translation of radiotracers for positron emission tomography/single-photon emission computed tomography (PET/SPECT). Since the discovery of GLUT family proteins overexpression associated with certain tumors, a variety of radiolabeled glucose derivatives have been developed as SPECT and PET tumor imaging agents. [18F]FDG is by far the most widely used in PET imaging for cancer diagnosis. Unfortunately, clinical usage is limited due to the need for the presence of cyclotron in 18F production. Generator produced isotopes, such as 99mTc and 68Ga, are readily available and affordable. The availability of a generator and kit chemistry to prepare 99mTc and 68Ga-based molecular probes may have a significant impact on nuclear medicine (Liu et al., 2014). It has been shown that 99mTc-glucarate may behave as a suitable alternative to 18F-FDG as a promising breast tumor imaging agent and needs to be further investigated (Gambini et al., 2011). Thus, using generator-produced isotopes to label glucose analogs is the major focus of ongoing research.
4. Identification and assessment of using imaging biomarkers currently associated with other cancer indicators in additional hallmarks such as hypoxia, invasion and changes in metabolism. During the past decades, researchers have tried to elucidate the mechanisms that underlie cancer-related death. However, this remains a challenge, as genomic instability causes a constantly changing genetic profile of tumors, and local variations in the microenvironment cause heterogeneity in tumor cell behavior (Polyak, 2014).
5. How to validate novel imaging biomarkers in adequately powered multi-center clinical trials. While applied molecular biology to cancer has made great advancements, the development of clinically validated biomarkers for primary breast cancer has remained an unconquerable task. Chemo-N0 (1993–1998) was the first prospective randomized multicenter trial in Node-negative breast cancer designed to prospectively evaluate the clinical utility of a biomarker. Its results established uPA/PAI-1 as a clinically useful biomarker for assessing long-term prognosis in early breast cancer and benefit from adjuvant chemotherapy in the high-risk group; it is thus well-suited for routine risk assessment in node-negative breast cancer (Harbeck et al., 2013). The Node Negative Breast Cancer Trial (NNBC), initiated by the Swedish Breast Cancer Group, was able to validate a prognostic index consisting of a proliferation factor, PR-status, and tumor size. The index may be helpful for prognostic considerations and for selection of patients in need of adjuvant therapy (Klintman et al., 2013). Although still in their infancy, circulating mi-RNAs and cf-DNA are beginning to be recognized as vital to future strategies on therapies for breast cancer (Ng et al., 2013; Esposito et al., 2014). mi-RNAs have become the rising stars for novel molecular targeting treatments because of their ability to regulate multiple genes in molecular pathways (Si et al., 2013). Very recently, a Phase 1 clinical study of MRX34, the first miRNA to advance into a human clinical trial for liver cancer, was approved (Mirna Therapeutics, 2014).
6. Methods of reporting intratumoral heterogeneity and locate the most beneficial areas for biopsies and radiotherapy. Within the plethora of imaging modalities, diffusion weighted magnetic resonance imaging (DW-MRI) has shown promise for the detection and characterization of breast cancer. Apparent diffusion coefficient (ADC) values allow quantification of the diffusion signal, and can facilitate in differentiating benign and malignant breast tumors as well as identifying early response in tumors undergoing preoperative treatment (Partridge and McDonald, 2013 and the references cited therein). On the other hand, the heterogeneous nature of cancer still presents an important challenge in cancer imaging and therapeutics (Seoane and De Mattos-Arruda, 2014). This heterogeneity also confers to the different breast cancer subtypes a specific invasional kinetic pattern, as has been recently shown by Yamaguchi et al. using MRI studies (Yamaguchi et al., 2014). Although breast MRI accuracy for assessing residual disease is good and surpasses other diagnostic techniques, overestimation and underestimation of residual disease have also been observed. This is largely because of the various treatment types and breast cancer subtypes (Lobbes et al., 2013). With some limitations and taking into consideration that many of the experiments have been performed in mice, intravital microscopy (IVM) is another technique that has proven its power to elucidate the cellular and molecular events that underlie the hallmarks of cancer. Fluorescence-guided surgical procedures have also benefited from IVM, which translates into more promising uses in the clinical setting (Ellenbroek and Van Rheenen, 2014).
7. Extension of methods that identify and define subtypes of cancerous tumors —DCIS, TNBC and luminal types—with non-invasive procedures (which may identify mixed lesions missed by homogenized or limited sample analyses) and assess heterogeneity between metastases. In recent years, there has been an explosion in the field of nanomedicine with the development of new nanoparticles for the diagnosis and treatment of cancer, and the related term "nanooncology" has been adopted by many (Thakor and Gambhir, 2013). The development of new contrast agents for MRI opens a new way for non-invasive breast cancer characterization. Special attention is made to iron oxide nanoparticles, currently one of the best options in clinic due to its lack of toxicity (Kievit and Zhang, 2011; Rosen et al., 2012). Meier et al. used magnetic nanoparticles (SPIONs) decorated with folic acid to image FR-positive human breast cancer cell lines non-invasively (Meier et al., 2010). Very recently, Sun et al. have developed SPIONs functionalized with extra domain-B of fibronectin (EDB-FN)

peptide for *in vivo* imaging of breast tumor initiating cells (BTICs) by MRI (Sun et al., 2014). The development of new algorithms in contrast enhanced MRI is also enabling good discrimination of triple-negative cancers from non–triple-negative cancers, as well as between triple-negative cancers and benign fibroadenomas (Agner et al., 2014). This computer assisted diagnosis opens a new window for quick breast cancer identification and therapeutical match.

8. Combination of multidisciplinary inter-field data. It has been recommended that imaging studies (both preclinical and clinical) would need to be coregistered with linked genomic and proteomic information in order to fully understand the biological implications of the images registered (Segal et al., 2007; Lambin et al., 2012; Waterton and Pylkkanen, 2012). Currently, imaging studies are often separated from tissue collection due to a lack of appreciation of how the coordination could benefit.
9. Identification and evaluation of biomarkers with therapeutic responses. More extensive usage of orthotopic xenograft and transgenic murine models of primary and metastatic breast cancer will demand robust preclinical imaging approaches. Trials that make use of these images will experience increased accuracy for novel agents, which in turn can speed up the development of successful treatments and the early cessation of those that show no promise (publication of negative results has been recommended by many researchers Alcantara et al., 2010; Anderson et al., 2013). These preclinical trials might also lead to sequential and combination treatment regimens.

As has been listed above, there are many new and emerging molecular imaging technologies that can benefit breast cancer patients. Other molecular imaging procedures under development often combine imaging systems to form hybrid technologies that improve accuracy and allow physicians to see how cancer may be affecting other systems in the body. One of the more promising research areas is in investigational PET imaging biomarkers, such as fluorothymidine (FLT) and fluoroestrogen (FES). FLT has shown promise for the demonstration of tumor proliferation and FES for the demonstration of estrogen receptors. Other exciting area of study is radioimmunotherapy, a form of treatment that targets cancer-killing radiation directly to cancer cells (out of the scope of this review).

CONCLUSIONS

The past 40 years have seen stunning improvements in the ability of noninvasive imaging to characterize structures and functions. These strides have come from the progressive evolution of conventional imaging techniques, with relatively little impact from imaging targeted to specific molecular moieties. Although the basic science of molecular imaging continues to make impressive strides, the regulatory and commercial landscape is limiting to these investigational imaging agents.

We anticipate that future needs will include the development of nanomaterials that are specific for immune cell subsets and can be used as imaging surrogates for nanotherapeutics. New *in vivo* imaging clinical tools for noninvasive macrophage quantification are thus ultimately expected to become relevant to predicting patients' clinical outcome, defining treatment options and monitoring responses to therapy.

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A biological perspective toward the interaction of theranostic nanoparticles with the bloodstream – what needs to be considered?

Martin J. D. Clift[1]*, Jean-François Dechézelles[1], Barbara Rothen-Rutishauser[1] and Alke Petri-Fink[1,2]

[1] BioNanomaterials, Adolphe Merkle Institute, University of Fribourg, Fribourg, Switzerland
[2] Department of Chemistry, University of Fribourg, Fribourg, Switzerland
*Correspondence: martin.clift@unifr.ch

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João Conde, Massachusetts Institute of Technology, USA

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Nanomedicine intends to create and further use novel materials at the nanoscale in order to provide an improvement upon current medical applications for human healthcare (ESF, 2005; Etheridge et al., 2013). In line with the advances made within nanotechnology since the late twentieth century (Mamalis, 2007) nanomedicine has received heightened attention due to its potential advantages, most notably within (cancer) theranostics (Muthu et al., 2014). The field of theranostics aims to utilize the physico-chemical characteristics of nanosized materials in order to intensify the effectiveness in diagnosing and treating diseases at the molecular level (Kim et al., 2013). Such a perspective is notably paramount for cancer types that are difficult to identify as well as apply therapy toward (e.g., secondary cancer) (Muthu et al., 2014).

Despite the well documented and proposed benefits of therapeutics in the nanosize range (Krol et al., 2013), for the majority of nanoparticles (NPs) [defined as "*a nano-object with all three dimensions in the nanoscale (1–100 nm)*" (BSI, 2007; ISO 27687, 2008)], the ability to merge the expansive divide between developing a significant advancement within material science and creating a biologically relevant therapeutic has proven to be a highly non-trivial task. One important reason for this is the relatively limited specific understanding of the biological interaction of therapeutic NPs following their administration into the human body and their subsequent delivery to the target site (e.g., tumor) (Capco and Chen, 2014). The objective of this opinion article therefore, is to provide a biological perspective upon what must be considered in the development of theranostic NPs.

WHERE SHOULD FOCUS BE GIVEN?

For biologically effective theranostic NPs, determining their dispersity, biocompatibility and biostability within different biological environments is imperative. For this, an understanding of the dynamic interaction between NPs with liquid and cellular systems as well as their subsequent biological impact must be gained. This outlook is not straight-forward and requires an intensive, multi-interdisciplinary research focus with cross-talk/feedback loops between the material scientists developing the materials and the biologists/clinicians wishing to study/apply them.

Initially, from a material perspective, there are an abundance of complex hurdles that must be overcome when developing any proposed nanotheranostic (Petros and DeSimone, 2010). Whether the NPs are manufactured for use as a treatment e.g., degenerative disease states (e.g., Alzheimer disease) (Liu et al., 2005), infectious diseases (e.g., hepatitis B) (Li et al., 2010) cancer (McMillan et al., 2014), or as a diagnostic tool (Niemirowicz et al., 2012), a systematic chemistry approach must be used (Davis et al., 2008). Whilst the specific shape of the NPs is of extreme interest regarding their efficiency as a theranostic agent (Liu et al., 2012), it is the precise material applied that is important, as well as the surface layer and the subsequent surface attachment of therapeutic agents and additional molecules (e.g., fluorophores, receptor-targetting moieties) to the modality (Petros and DeSimone, 2010), while keeping within the nanosize range. Additionally, determining their dispersity (i.e., colloidal stability) and biostability can also be laborious and problematic (Petros and DeSimone, 2010). Although these issues are not trivial, once the NP is engineered and ready for use, one of the main, biologically-based obstacles is to determine the ease of directing this modality to the site of interest within the human body without causing any undesirable effects (e.g., recognition and/or clearance by the immune system).

Successful targeting of theranostic NPs is an onerous concept (Nicolaides et al., 2014), and is commonly overlooked in favor of immediately focussing upon the effectiveness of the theranostic agent upon the specific target site (i.e., cancer cells for cancer therapeutics) (Xie et al., 2011). For example, a plethora of studies have been published which have shown the effectiveness of theranostic NPs in either the delivery of a drug to cells (Najafi et al., 2014), or destructing cancer cells with or without external stimuli (e.g., light, magnetic field) (Hayashi et al., 2014). Naturally, this approach is of extreme importance, and absolutely vital toward the development of any theranostic based NPs. However, the precise effective nature (i.e., efficacy) of the NPs upon the chosen target site can be considered as inextricably linked to the efficient transport of NPs from their administration site into the human body

to the specifically chosen target site (Davis et al., 2008). Therefore it is essential that the interpretation of the effectiveness of theranostic NPs directly upon the target site is considered after gaining a controlled understanding of the biological impact upon the NPs following their transport through different biological environments to said target site.

Thus, in order to fully elucidate the impact of the transport processes following administration of theranostic NPs until reaching their target site, comprehension as to what biological entities interact with the NPs, how their physico-chemical characteristics may be altered over time and from interaction with different environments, as well as subsequently how these potential adaptations might affect the effectiveness of the NPs when they engage with the intended site of interest is decidedly necessary. Focus upon these key aspects would further enable the enhanced development of theranostic NPs from a materials' perspective, allowing them to be optimized for maximal benefit toward their proposed application. Through this approach, significant improvement to the efficacy of the NPs to the target site would be obtained concomitantly. Nonetheless, which route of transport toward the target site of the applied theranostic NPs should be studied first?

FIGURE 1 | Schematic of a human blood vessel, representative of the human bloodstream. Image shows the three main cell types, (i) erythrocytes, also known as red blood cells (RBCs) (*represented as flat, disc-shaped red cells*), which contain hemoglobin (an iron-containing biomolecule responsible for oxygen (O_2) binding), (ii) leukocytes (i.e., white blood cells) (*represented as white, round cells*) and (iii) thrombocytes (i.e., platelets) (*represented as small purple cells*), the essential cell type that allows for blood clotting (Abbas and Lichtman, 2003). The human bloodstream is responsible for the circulation of nutrients (i.e., amino acids), O_2 and hormones, in addition to the removal of metabolic waste (e.g., carbon dioxide) (Abbas and Lichtman, 2003). It assists in regulating body temperature and pH, and further engages in the fighting of disease states. All of these functions contribute toward the essential maintenance of the homeostasis of the human body. In addition the human bloodstream is suspended within a protein matrix, abundant in albumin, known as plasma (i.e., blood serum together with fibrinogens) acting under the influence of non-classical hydrodynamic flow, known as haemodynamics (Abbas and Lichtman, 2003). The suitability of this holistic environment upon nanoparticles (*represented as gold spheres*) for theranostic applications is currently limited.

APPROACHING THE PROBLEM

The main administration route for most theranostic-based NPs is *via* intravenous injection (Nichols and Bae, 2012). Thus, the initial biological environment that these theranostic-based NPs will encounter is the complex cellular and molecular milieu of the human blood circulation (as described in **Figure 1**). Thus, foremost direction toward understanding its impact upon theranostic NPs is paramount.

To systematically study the impact of the bloodstream upon thernostic NPs, *in vivo* (i.e., rodents) assessment would rapidly determine the efficacy of NPs formulated for theranostics. Yet, despite encompassing a "whole-body" scenario, it would not provide species specificity, which would be necessary for the inevitable application of NPs as theranostic agents. Primates would therefore be ideal, as used in the study by Ye et al. (2012), who showed the applicability of quantum dots as useful theranostic tools. However, neither *in vivo* strategy would provide the basis for a systematic study as to how NPs may interact with their numerous local environments (i.e., within the bloodstream) prior to arriving at their intended target site in the human body. By adopting an *in vitro* perspective however, it would enable a controlled outlook toward studying the impact of each biological constituent of the human bloodstream upon the chosen theranostic NPs. Difficulties in this approach arise however, since it would only allow for monoculture, or, at the most, co-culture systems to be used to conduct such investigations. Although advanced *in vitro* systems concerning the bloodstream and its constituent parts are being established, such as the advanced platelet model system recently reported by Thon et al. (2014), a finite model system that mimics the bloodstream is currently lacking. Therefore, currently, to comprehend how biological environments, such as the bloodstream, may impact upon the effectiveness of theranostic NPs a combined *in vitro* and *in vivo* approach should be integrated as a vital component in the development of theranostic NPs.

On an additional note, it is prudent to note that such a systematic study of any therapeutic NPs from the specific exposure site, via the potential transport route to the target site should be performed in order to gauge their potential effectiveness following administration. In this

regard, it is also relevant to highlight that a series of other exposure routes, including ingestion, cutaneous and inhalation (Melancon et al., 2012), the latter for which theranostic applications are being derived (Pison et al., 2006), also pose a potential access route for NPs into the blood circulation *via* translocation across cellular barriers (Kreyling et al., 2012). Furthermore, the use of NPs to coat implants (i.e., for antimicrobial purposes) has recently increased (Kempe et al., 2010), and therefore it is possible that these could further concentrate the NPs gaining access into the human bloodstream, also *via* barrier cell translocation. Yet, the presence of NPs within the bloodstream from these exposure routes represents a secondary, non-specific exposure scenario and relates to a risk perspective. Whilst risk assessment is not the purpose of this article, it is worth to highlight that this issue has received limited attention to date, and requires further, in-depth investigation which could advantageously coincide with the advancement of NPs for nanomedicine-based applications (i.e., understanding their biocompatibility).

MOVING FORWARD

Due to the lack of an advanced *in vitro* model system, as previously highlighted, determining the role of each component of the bloodstream as to its potential impact upon theranostic NPs is imperative to their overall development. However which constituents are important?

Most notably, the immediate and abundant adherence of proteins (as well as lipids) to the surface of any theranostic NPs entering the bloodstream (Lynch et al., 2006) can create a possible issue towards the surface molecules attached for a specific therapeutic purpose (i.e., receptor-binding sequence), as well as a loss in colloidal stability due to aggregation (Hirsch et al., 2014). Although NPs with varying physico-chemical characteristics can be manipulated for nanotheranostics, it has become abundantly apparent that similar proteins are consistently found upon the surface of NPs independent of their surface coating/charge (Hirsch et al., 2013). Whilst this is a dynamic process upon the surface of NPs, there remains a hard protein layer on top of the NPs at all times, thus posing a significant issue to material scientists. Yet, if coated with abundant proteins, these can engage with the epitopes on the immune cells, and so it is difficult to decipher if the steric repulsive barrier of a polymer shell would still remain effective enough to prevent uptake by these phagocytic cells, or not. Although, if internalized by the immune system, will they be processed and potentially exocytosed by these cell types, and exhibit the same properties prior to their administration? What the physico-chemical state of the NPs is following this interaction is currently unknown, and requires in-depth investigation. If however, the immune system does not recognize the NPs, then there is a heightened possibility that they could pass, unimpeded into erythrocytes (Rothen-Rutishauser et al., 2006). The impact that this cellular interaction may have upon the NPs is relatively unknown. Although if the NPs become present within these cell types, circulation time (of the NPs) will most likely increase, perhaps rendering them ineffective and/or aggregating within the bloodstream with potential adverse/fatal consequences in the long-term. In addition to these cellular/molecule based issues, the effect of the injection process (e.g., pressure, flowrate, pH and temperature changes) upon the physico-chemical characteristics of the NPs *via* their administration route must also be conceived. Therefore, increased research strategies must be directed toward this approach to achieve the successful development of theranostic NPs.

OVERALL PERSPECTIVE

Due to their inevitable administration to the human body via intravenous injection, understanding of the interaction of theranostic NPs with the complex biological environment of the bloodstream is vital in regards to their development. The knowledge created from this approach could enable key understanding to be gained as to the ability for the NPs to withstand the confines of this local environment. Furthermore, it will provide imperative insight into their ability to effectively perform the task they were engineered to achieve (e.g., drug delivery). Since following this approach the NPs will most likely require further manipulation regarding their physical and chemical characteristics, in order to achieve this outlook an enhanced, multi-interdisciplinary approach must be adopted. By combining the expertise of a variety of disciplines it will enable the advancement of systematic studies of the physical and chemical state of the NPs based on the impact observed when NPs are present within the bloodstream. Therefore, this perspective will facilitate the essential development required to successfully manufacture effective theranostic NPs for human health care.

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Multifunctional gold nanostars for molecular imaging and cancer therapy

Yang Liu[1,2,3], *Hsiangkuo Yuan*[1,2], *Andrew M. Fales*[1,2], *Janna K. Register*[1,2] and *Tuan Vo-Dinh*[1,2,3]*

[1] Fitzpatrick Institute for Photonics, Duke University, Durham, NC, USA, [2] Department of Biomedical Engineering, Duke University, Durham, NC, USA, [3] Department of Chemistry, Duke University, Durham, NC, USA

Plasmonics-active gold nanoparticles offer excellent potential in molecular imaging and cancer therapy. Among them, gold nanostars (AuNS) exhibit cross-platform flexibility as multimodal contrast agents for macroscopic X-ray computer tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), as well as nanoprobes for photoacoustic tomography (PAT), two-photon photoluminescence (TPL), and surface-enhanced Raman spectroscopy (SERS). Their surfactant-free surface enables versatile functionalization to enhance cancer targeting, and allow triggered drug release. AuNS can also be used as an efficient platform for drug carrying, photothermal therapy, and photodynamic therapy (PDT). This review paper presents the latest progress regarding AuNS as a promising nanoplatform for cancer nanotheranostics. Future research directions with AuNS for biomedical applications will also be discussed.

Keywords: multifunctional, plasmonics, gold nanostars, cancer imaging, cancer therapy

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***Correspondence:**
Tuan Vo-Dinh, Department of Biomedical Engineering, Duke University, Durham, NC 27708, USA
tuan.vodinh@duke.edu

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Introduction

Nanotheranostics, combining diagnostic and therapeutic functions into one nanoparticle, has attracted great attention due to its promise as a powerful tool for personalized therapy to treat cancer, which contributes to more than seven million deaths each year (Ferlay et al., 2010; Jemal et al., 2010; Wang et al., 2014). Theranostic nanoprobes can potentially be used for image-guided cancer therapy with improved targeting specificity and therapeutic efficacy (Choi et al., 2012). Nanoparticles of sizes ranging from 20 to 100 nm accumulate in tumors through the well-known enhanced permeability and retention (EPR) effect that originates from the leaky tumor vasculature (Maeda, 2001; Maeda et al., 2001, 2003; Sykes et al., 2014). Furthermore, targeting ligands, including antibodies and peptides, can be functionalized on nanoparticles for active cancer targeting (Kim et al., 2011b). Various nanoplatforms, including liposomes, iron oxide nanoparticles, and gold nanoparticles have been developed for cancer imaging and treatment (Ahmad et al., 2010; Xie et al., 2010; Lee et al., 2014; Wang et al., 2014). In particular, gold nanoparticles have demonstrated great biocompatibility, plasmonic properties, and convenience in versatile functionalization through strong gold-thiol interactions (30–40 kcal/mol), as can be exemplified by gold nanorods, nanoshells, and nanocages in preclinical and clinical cancer management (Ramachandran et al., 2003; Loo et al., 2004; Alekseeva et al., 2006; Choi et al., 2012; Huschka et al., 2012; Xia et al., 2012b; Ahmadi and Arami, 2014; Lee et al., 2014; Xia and Xia, 2014). Those established plasmonic gold nanoplatforms (nanorods, nanoshells and nanocages) have been widely used in previous studies for *in vitro* and *in vivo* investigations of cancer imaging with positron emission tomography (PET), magnetic

resonance imaging (MRI), X-ray computer tomography (CT), high resolution optical imaging with labeled fluorescence dyes as well as photothermal therapy (PTT) and photodynamic therapy (PDT). Furthermore, gold nanospheres and nanoshells have been used in clinical trials for drug delivery and photothermal therapy, respectively (Gad et al., 2012). Therefore, novel nanoplatforms are of great interest for cancer detection and treatment.

Gold nanostars (AuNS), with multiple sharp branches (**Figure 1A**), have superior tip-enhanced plasmonic properties in the near-infrared (NIR) tissue optical window, which is suitable for *in vivo* biomedical applications. Plasmonic AuNS have been applied for *in vivo* lymphatic system mapping with photoacoustic tomography (PAT) (Kim et al., 2011a). AuNS with silica shells have been found to be internalized into living cells and can be used for intracellular imaging (Rodriguez-Lorenzo et al., 2011; Fales et al., 2013; Yuan et al., 2013a). In addition, AuNS surface-enhanced Raman scattering (SERS) nanoprobes have been applied for immuno-SERS microscopy of the tumor suppressor p63, imaged in prostate biopsies (Schutz et al., 2011). SERS takes advantage of a unique phenomenon on certain metal nanoparticles, surface plasmon resonance (SPR). Surface plasmon refers to oscillating electrons within the conduction band when the metallic nanostructure surface is excited by an external electromagnetic field. The oscillating electrons can generate a secondary electromagnetic field, which is added to the external electromagnetic field to result in SPR. Incident photon energy, when in resonance with the surface plasmon, magnifies the local electromagnetic field that dramatically enhances the intrinsically weak Raman signal. The SERS enhancement factor is typically 10^6–10^8-fold and can be up to 10^{15}-fold in hot spots, where the electromagnetic field is extremely intense (Liu et al., 2015b). By combining resonance enhancement, surface-enhanced resonance Raman scattering (SERRS) shows even greater Raman signal enhancement than SERS alone. Silica-coated AuNS SERRS nanoprobes have been used for visualizing brain tumor margins and microscopic tumor invasion (Harmsen et al., 2015). Our group has developed a novel toxic surfactant-free AuNS synthesis method that greatly improves the biocompatibility and the versatility of surface functionalization (Yuan et al., 2012b). In this review paper, we will focus on the latest progress achieved in our laboratory related to AuNS and discuss their bright future for theranostic applications.

Multimodal Imaging

AuNS provide a powerful tool for 3D *in vivo* tracking and disease detection with whole body scans. For CT imaging, gold nanoparticles are superior to traditional iodinated contrast agents, since gold has a higher atomic number ($Z = 79$) and k-edge value (80.7 keV). In addition, the X-ray attenuation of iodine is decreased by water while that of gold is not. Gold nanoparticles can absorb X-ray, which subsequently generate photoelectric and Compton effects. These effects lead to formation of secondary electrons and reactive oxygen species (ROS), the yield of which correlates with the surface area of nanoparticles (Misawa and Takahashi, 2011). Since star-shaped geometry exhibits greater surface area than the spherical counterpart of equivalent size, AuNS can be an improved sensitizer for radiation therapy. For MRI imaging, Gd^{3+} ions have been linked to the AuNS surface through 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelator. As shown in **Figure 1B**, tumor cells loaded with multifunctional AuNS probes display high intensity under CT and MRI examination (Liu et al., 2013a). PET scan provides an extremely sensitive 3D imaging method. The sensitivity could reach picomolar for PET compared to micromolar for MRI. We performed PET imaging with ^{64}Cu-labeled AuNS nanoprobes for dynamic imaging up to 24 h (**Figure 1D**). *In vivo* tracking results showed that the developed AuNS nanoprobes accumulate gradually in tumor with 3.3:1 tumor-to-muscle ratio at the end of 24 h (Liu et al., 2015b). These studies exemplify the potential of AuNS for whole body imaging.

In addition, AuNS have been exploited as a powerful optical contrast agent under multiphoton microscope for high-resolution imaging. With an exceptionally high two-photon photoluminescence (TPL) [more than one million Göeppert-Mayer (GM) two-photon action cross-section (TPACS)], AuNS offer superior signal contrast than quantum dots or other organic fluorophores for multiphoton optical imaging (Yuan et al., 2012b). Furthermore, a recent study investigated TPL of single gold nanoparticle with different shapes and the TPACS were reported to be 83,500, 1.5×10^3, 4.2×10^4, 4.0×10^6 GM for nanosphere, nanocube, nanotriangle, nanorod and nanostar, respectively (Gao et al., 2014). The TPACS of AuNS is almost two orders higher than that of gold nanorod, which is the highest one among other shapes. With an intense TPL emission, AuNS can be used not only for real-time *in vivo* tracking, but also for sensitive tumor detection following systemic injection of AuNS (Yuan et al., 2012c). As shown from TPL imaging in **Figure 1C**, transactivator of transcription (TAT)-functionalized AuNS nanoprobes have much stronger signal in cells than that of AuNS without TAT functionalization. TAT is a well-known cell-penetrating peptide with capability to increase nanoparticle cellular uptake. Although the TPL imaging depth is limited by the extent of laser penetration, optical microscopy offers the highest spatial resolution among most animal imaging modalities. AuNS are also suitable to be used under PAT with high extinction coefficient ($\sim 10^{10}$ $M^{-1}cm^{-1}$) (Xia et al., 2012a; Yuan et al., 2014). Being a potent contrast agent for PAT, which is an optical-ultrasound hybrid imaging technology, the particokinetics and biodistribution of the AuNS can be studied with deeper imaging depth and larger field of view.

Furthermore, AuNS have been applied for SERS detection with tip-enhanced plasmonics. The reported SERS enhancement factor is several orders higher than that of gold nanospheres (Yuan et al., 2013a). SERS applies nanometallic structures to enhance "fingerprint" Raman spectra, which provides a method for molecular sensing with high sensitivity (Liu and Sun, 2011; Liu et al., 2013b,c; Yuan et al., 2013b; Zhao et al., 2014a). Our group demonstrated the first analytical application of SERS in chemical analysis using nanostructured metal substrates 30 years ago, and has been working on various types of SERS nanoplatforms, including nanogratings, nanorod arrays, nanowires and AuNS, for biochemical sensing (Meier et al., 1985;

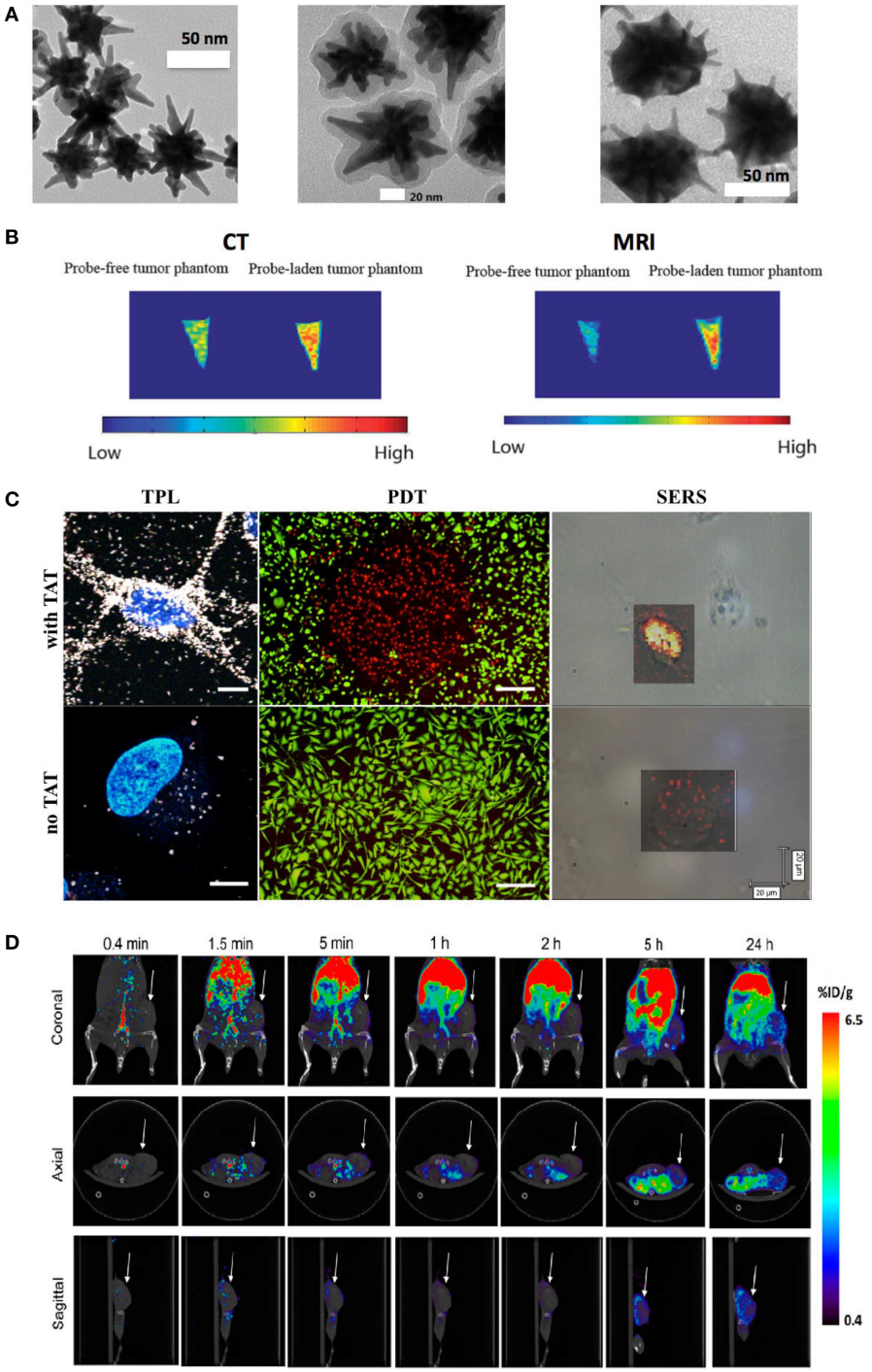

FIGURE 1 | (A) TEM images of synthesized AuNS (left), silica-coated AuNS (middle), and silver coated-AuNS (right). **(B)** CT (left) and MRI (right) imaging with developed multifunctional AuNS nanoprobe. **(C)** Demonstration of TAT-peptide enhanced TPL imaging, photodynamic therapy (PDT), and surface-enhanced Raman scattering (SERS) imaging after 1-h incubation with the AuNS probe. Scale bars for TPL, PDT and SERS image are 10, 250, and 20 μm, respectively. **(D)** PET/CT imaging with ^{64}Cu labeled AuNS probe, which was injected intravenously through tail vein. Dynamic imaging at various time pointes were acquired. Arrow shows tumor (Fales et al., 2011, 2013, 2014; Yuan et al., 2013a; Liu et al., 2015b).

Enlow et al., 1986; Alak and Vo-Dinh, 1987; Bello et al., 1989; Vo-Dinh et al., 1994, 2013; Stokes et al., 2004; Yuan et al., 2013a). SERRS AuNS nanoprobes have been developed to perform quantitative *ex vivo* multiplex detection with high sensitivity (Yuan et al., 2013b). The AuNS nanoprobes have also been used for SERS imaging for cancer cells as shown in **Figure 1C** (Fales

et al., 2013). SERS images were acquired at 633 nm with a 2-μm step size by using a Renishaw Raman microscope. The 633 nm resonant dye, DTDC was used to label the AuNS for SERS detection. SERS imaging shows that TAT-functionalized AuNS nanoprobes have much stronger signal inside cells. Recently, we have reported the synthesis of silver-coated AuNS (AuNS@Ag) that provide over an order of magnitude increase in SERS signal when compared to AuNS alone (Fales et al., 2014). We have applied the AuNS@Ag for *in vitro* homogenous nucleic acid detection, which demonstrated AuNS' strong capability for molecular sensing with the SERS method (Wang et al., 2015). With such flexibility, AuNS thus are a promising nanoplatform for multimodality imaging from whole body scan to high-resolution optical imaging as well as molecular sensing.

Brain Tumor Imaging

The potential of AuNS as an efficient contrast agent has further been investigated in animal brain tumor models (Yuan et al., 2014). Through cranial window chambers implanted with tumor cells in live animals, AuNS have been employed for angiography with high spatial resolution; cerebral capillaries were clearly visible with minimal tissue autofluorescence background. Unlike conventional contrast agents (e.g., FITC-dextran) showing signal decay within 30 min, AuNS can be coated with polyethylene glycol (PEG) to achieve a much longer intravascular signal stability due to its extended serum half-life (several hours) and lower degree of extravasation.

The main challenge of tumor imaging is delivering the contrast sufficiently to the target. Several physiological barriers exist between the injection site and the target tumor cells. For example, nanoparticles need to survive immunoclearance, extravasate tumor vessels, permeate the blood brain barrier (BBB), diffuse through interstitium, and penetrate the plasma membrane into cells (Chrastina et al., 2011; Goldberg et al., 2013). For brain tumor imaging, the greatest challenge lies in the permeation of the BBB. Tight junctions between the endothelial cells and podocytes from the astrocytes form a highly selective barrier that prevents large molecules from passing through. To date, the typical 24-h post-injection brain accumulation of nanoparticles may still be less than 0.1% of the initial dosage (Khlebtsov and Dykman, 2011); this is similar to those obtained after monoclonal antibody infusion. Several nanoparticle-based platforms have been studied, including transferrin-containing gold nanoparticles (Wiley et al., 2013), polysorbate 80-coated poly(n-butyl cyanoacrylate) dextran polymers (Koffie et al., 2011), and angiopep2 peptide-functionalized dendrimers (Yan et al., 2012). Since AuNS are a strong contrast agent, it is therefore prudent to investigate the effect of surface functionalization on brain tumor targeting.

Nanoparticles accumulate in tumors typically through passive and active mechanisms. While surface PEGylation achieves the former, the latter requires surface functionalization of peptides or proteins. Upon systemic exposure, PEGylated AuNS accumulate passively near the tumor periphery and around blood vessels. Hyper-neovascularity along the tumor edge and interstitial fluid pressure gradient at the boundary attenuate the penetration of AuNS deep into the tumor. PEG-AuNS also accumulate in vascular endothelial cells but with minimal true transcytosis(Ye et al., 2013), where paracellular extravasation is most likely attributed to the defective tight junction at the tumor site. With extravasation depending on the nanoparticle size and incubation time (Yuan et al., 1994; Popovic et al., 2010), smaller nanoparticles or longer incubation may further increase the tumor accumulation or extravasation depth.

Comparing the brain tumor AuNS delivery through passive and active mechanisms, we coated 80-nm AuNS with PEG (PEG-AuNS) and TAT peptides (TAT-AuNS), and 50-nm AuNS with angiopep2 peptides (angiopep2-AuNS). Angiopep2-peptide has recently been exploited to deliver nano-drugs into the brain *via* lipoprotein receptor related protein (Gabathuler, 2010). PEG-AuNS accumulated in the tumor periphery and parenchyma but much less in the normal brain. They also accumulated inside endothelial cells and perivascular spaces, particularly in the tumor regions of defective vascular integrity. As shown in **Figure 2**, TAT-AuNS accumulated less in tumor but extensively in liver compared to PEG-AuNS. Positively charged TAT possibly attracted opsonins, causing greater entrapment in the reticuloendothelial system. In contrast, smaller angiopep2-AuNS had a deeper distribution inside tumor parenchyma with relatively less liver accumulation. Calculated from the AuNS intensity ratio from the images collected under the same microscopic settings, the average tumor/liver AuNS density ratios are 0.32, 0.03 and 1.2 for PEG-, TAT- and angiopep2-coated AuNS. These findings suggest that enhanced tumor BBB permeation with selected intratumoral delivery can be achieved with proper control of AuNS sizes and selection of surface ligand chemistry.

AuNS for Phototherapy

AuNS have also been applied for PDT and photothermal therapy (PTT). PDT uses a photosensitizer that generates ROS upon irradiation to kill cells, while photothermal therapy transduces light to heat for cancer hyperthermia or ablation. The effect of laser type on PTT has been investigated before and the results showed that pulsed lasers could use 10 times less energy to kill cancer cells than that used for continuous-wavelength (CW) lasers (Huang and El-Sayed, 2011). That is because pulsed-laser with gold nanoparticles can lead to a variety of therapeutic effects including protein denaturation, cell cavitation, bubble formation from shock waves and plasma generation (Pustovalov et al., 2008). With the nanoparticle's tumor targeting effect and focused irradiation to the tumor site, phototherapy can achieve greater tumor therapeutic specificity with less off-target effects. The therapeutic potentials have been demonstrated on gold nanoshells, nanocages, and nanorods (Von Maltzahn et al., 2009; Xie et al., 2010; Day et al., 2011). Gold nanoshells were used to perform PTT (800-nm diode laser, 4 W/cm^2 intensity for 3 min.) with murine xenograft models and results showed significant survival rate improvement for the treatment group with 57% of mice remaining tumor free at the end of 90 days, while the mean survival date for the control group was only 13.3 days. Gold nanocages functionalized with HER2-antibody have

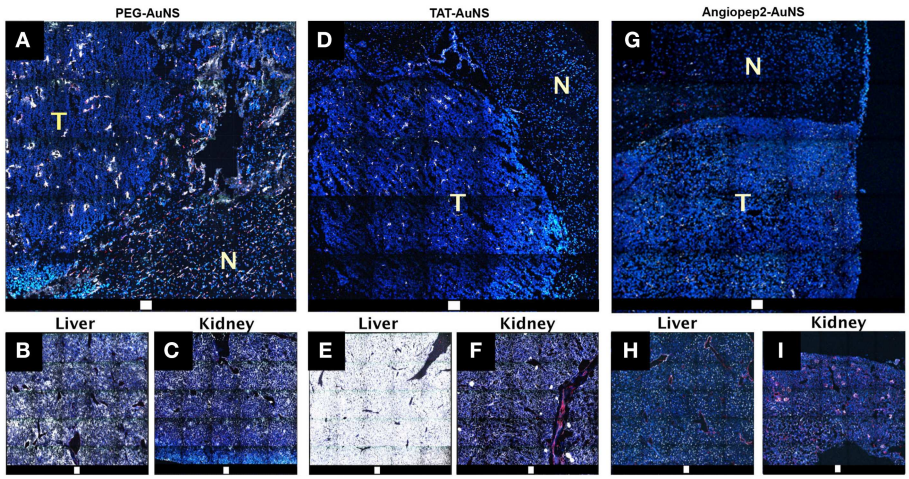

FIGURE 2 | TPL images of DAPI- (nucleus; blue) and CD31 (blood vessel; red)-stained cryosectioned specimens from perfused brain excised 48-hr after injection (5 pmole; 80 nm) of neutral PEG-AuNS (A–C) and positively-charged TAT-AuNS (D–F), and injection (1 pmole; 50 nm) of angiopep2-AuNS (G–I). Large-area tile images showing distribution of PEG-AuNS **(A)**, TAT-AuNS **(D)**, Angiopep2-AuNS **(G)** preferentially in the brain tumor than normal brain. T, tumor. N, normal. Charged TAT-AuNS accumulated greater in the liver hence lower intratumoral accumulation. Smaller angiopep2-AuNS was more widespread in the tumor. Scale bar: 100 μm (Yuan et al., 2014).

been used to perform PTT with a femtosecond Ti:Sapphire laser (810 nm, 1.5 W/cm^2 for 5 min) for SK-BR-3 breast cancer cells. The treatment group shows a majority of cells died while there was little cellular death in the control group (Skrabalak et al., 2007). Gold nanorods with T790 dye embedded in a silica shell were developed to perform PDT and the improved treatment efficiency has been demonstrated with HepG2 cancer cells (Zhao et al., 2014b). In particular, the star-shape geometry of AuNS generates a strong tip-enhanced plasmonic effect that greatly increases the photothermal transduction efficiency especially in the NIR tissue optical window. Successful photothermolysis has been demonstrated in both single photon and two photon settings (Yuan et al., 2012a,c). An ultralow irradiance of 0.2 W/cm^2 (at using 850 nm pulsed laser), which was below the maximal permissible exposure of skin, was reported. A recent study demonstrated that AuNS exhibit higher photothermal transduction efficiency than gold nanoshells (90–94% for AuNS compared to 61% for gold nanoshells). *In vivo* experiment also revealed a successful photothermal ablation of aggressive sarcoma tumor in mouse after AuNS systemical injection through tail vein (Liu et al., 2015a). In addition, we also loaded Protoporphyrin IX onto silica-coated AuNS for PDT showing specific cytotoxic effect only on the UV exposed region (**Figure 1C**) (Fales et al., 2013). These results demonstrated that AuNS have great potential for PDT and photothermal therapy in future preclinical cancer treatment studies.

Conclusion and Future Perspective

The AuNS synthesized with the surfactant-free method provide a novel nanoplatform for cancer nanotheranostics. The multifunctional AuNS nanoprobes can be used for future pre-treatment cancer diagnostics with PET, MRI, and CT scan, intraoperative imaging with TPL, PAT, and SERS, and image-guided therapy with PDT and PTT. The plasmonic AuNS is a promising theranostic nanoplatform since they have combined capabilities for diagnostics with various imaging modalities from whole-body to subcellular level, therapeutics with phototherapy and drug delivery, and surface functionalization versatility for cancer targeting. Compared to other gold plasmonic nanoplatforms, the AuNS nanoprobes have unique advantages of tip-enhanced plasmonics, high TPL cross-section, and excellent photon-to-heat conversion efficiency, toxic surfactant-free synthesis. Further studies on relationship between AuNS properties including particle size and surface charge, and their biodistribution are still required to improve nanoprobe uptake in tumor for better biomedical applications. With promising findings from previous studies, AuNS demonstrate great potential for translational medicine studies as a combined nanoplatform for both cancer imaging and therapy.

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