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RECEIVED 12 August 2024

ACCEPTED 21 April 2025

PUBLISHED 06 May 2025

## CITATION

Mota WS, Severino P, Kadian V, Rao R,  
Zielińska A, Silva AM, Mahant S and Souto EB  
(2025) Nanometrology: particle sizing and  
influence on the toxicological profile.  
*Front. Nanotechnol.* 7:1479464.  
doi: 10.3389/fnano.2025.1479464

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# Nanometrology: particle sizing and influence on the toxicological profile

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The *in vitro* and *in vivo* performance of nanoparticles is directly linked to their physicochemical attributes, i.e., their shape, size, crystal phase and surface properties. By definition, nanoparticles are particles with three external dimensions in the range of 1–100 nm, per ISO 80004-1:2023. They may be naturally occurring, incidental, or intentionally engineered, with their physicochemical properties influencing their biological interactions. Broadly, they have been classified as ultrafine nanoparticles if not intentionally produced, and as engineered nanoparticles produced in a systematic and controlled manner. The designation of “nano,” as affixed to particulate systems, is determined by the size of discrete particles. Therefore, rigorous analysis of particle size and accurate assessment of their properties with a special focus on their particle size distribution, morphology and surface chemistry, is of great importance for their interaction with the biological surroundings. In this review, we discuss the main analytical techniques used in particle size measurements and relate the outcomes with the cytotoxicity and genotoxic profile of nanoparticles commonly used for drug delivery.

## KEYWORDS

nanoparticles, particle sizing, nanotoxicity, cytotoxicity, genotoxicity, drug delivery

## 1 Introduction

In recent decades, nanotechnology has been leading the research and development in the field of drug delivery and targeting, giving birth to materials with unique physicochemical characteristics that offer distinct biological properties (Ramakrishna and Rao, 2011; Yusuf et al., 2023).

According to the US National Nanotechnology Initiative, nanotechnology is the study and manipulation of matter at sizes ranging from 1 to 100 nm, where special properties allow for new and innovative uses (Bayda et al., 2019). According to the international standard ISO 80004-1:2023, a nanoparticle (NP) is defined as a material whose three external dimensions (length, width and height) are in the range of 1–100 nm (ISO, 2023). When the nanomaterial is a nanowire or nanofilm, the definition refers to two dimensions and one dimension, respectively. Broadly, NPs have been categorized as ultrafine NPs when

not intentionally produced, and as engineered NPs if produced for a specific purpose (Gupta and Xie, 2018).

To be classified as “nano,” the accurate determination of their particle size and size distribution is required (Babick et al., 2016; Caputo et al., 2019). A systematic characterization of the particle size and polydispersity would also help to establish an authentic protocol for their quality control and quality-assurance, especially at the industrial level. More importantly, many of the crucial pharmaceutical (e.g., drug payload, dissolution and release profiles), pharmacokinetic and pharmacodynamic (e.g., half-life, drug distribution and interactions with cells and tissues) parameters of NPs and their stability are directly influenced by the particle size (Caputo et al., 2019; Krawczykowski, 2024). Aspects, such as dispersion, sedimentation, coagulation, light scattering and inertial phenomena, also depend on the particle motion. The motion of small dispersed NPs, in turn, is influenced by their size (Yahyaee et al., 2024). When it comes to the reporting of particle size, the average diameter is generally used, implying that such measurements are based on the assumption that NPs are spherical in shape. In fact, it is the simplest method, useful in the measurements of a monodisperse system (i.e., a system containing particles of uniform size and shape). However, NPs encountered in pharmaceutical applications are generally polydisperse, and particle size distribution analysis gives a measure of polydispersity of the sample (Mahant et al., 2019; Mahant et al., 2020). Based on the product quality requirements, size of particles may be described by its minimum or maximum length or its average diameter. A multitude of methods are available for the assessment of particle size, each based on a principle related to some property of the particle, such as surface area and sedimentation rate. In the context of nanosized particles, the volume is a significant parameter, along with the polydispersity index (Pdl), that is used to define product specifications.

Besides the principles of the instrument, other factors that influence particle size include pH, ionic strength and temperature of the suspending medium. In case particle size determination of a sample is performed using a microscopic technique, the result for the same sample will depend on the type of microscopic analysis. Image analyses are based on the number distribution of particles within a size range, while laser diffraction can provide the mean particle size as number, volume or mass distribution. Thus, understanding the principle of the technique and the calculations underlying the reported results is very important for correct

interpretation. Experts also emphasize that average particle size measurements should be performed separately after dispersion of NPs in biological media. The components of biological media have an enormous impact on aggregation and sedimentation behavior of the dispersed particles. Moreover, the complex environment of biological media can induce interactions between the NPs and the plasma proteins, generating a protein corona and modifying the NPs' functionality, besides the variations in ionic strength and pH (Bashiri et al., 2023). In this review, we provide an in-depth discussion of the relevant physicochemical techniques that are commonly used for NPs size characterization, a comparison of their advantages and limitations, and highlight the effects of size on the toxicity risk of a variety of particles when used in drug delivery.

## 2 Nanoparticles sizing techniques

The fundamental principles of metrology (science of measurement), comprises practical and theoretical aspects of measurement, such as, measurement standards and methods, precision and accuracy, traceability, calibration, measurement uncertainty, quality control. Certified reference materials (CRMs, standards) and reference units must be used to ensure validation of the data collected, so that measurement results are reliable and comparable to internationally recognized standards, as defined by the International System of Units (SI, *Système international d'unités*). CRMs are essential in nanoparticle characterization, providing traceability to SI units and ensuring measurement accuracy by minimizing systematic bias. Measurement traceability is the property of a measurement result that enables its relation to a reference standard through a continuous chain of documented calibrations, each with a stated measurement uncertainty, ensuring comparability across laboratories which enables confidence in the result accuracy. While accuracy indicates how close a measured value is to the true value, precision indicates how close the measurement results are to each other (i.e., indicates the degree of repeatability or reproducibility). On the other hand, measurement uncertainty gives the range of values within which the true value of a measured quantity is estimated to be. The standards must be certified reference materials to provide reliable calibration and verification (Feltin et al., 2023).

Standard methods for NPs analysis are essential to ensure precision in nanoscale analyses and to guarantee the reproducibility of results. As example the Monte Carlo method that involves computational simulations based on repeated random sampling, is commonly used for high statistical accuracy (Carne et al., 2023). Thus, applying metrological principles to the measurement methods and techniques used to assess materials properties at the nanoscale (nanometrology) makes these measurements precise, reliable and traceable. Thus, advances in NP characterization and measurement methods contribute to the validity and quality of the results, allowing reliability of nanoscience studies.

### 2.1 Dynamic light scattering (DLS)

Dynamic light scattering (DLS), through detection of light scattering from matter, allows the measure of shape, size and size

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**Abbreviations:** AFM, Atomic Force Microscopy; BF-TEM, Bright-Field Transmission Electron Microscopy; CdSe, Cadmium selenide; CLSM, Confocal Laser Scanning Microscopy; Cryo-TEM, Cryo-Transmission Electron Microscopy; DCS, Differential Centrifugal Sedimentation; DLS, Dynamic Light Scattering; DSLs, Disodium Laureth Sulfosuccinate; EPR, Enhanced Permeability and Retention; EXAFS, Extended X-ray Absorption Fine Structure; FMR, Ferromagnetic Resonance; ICP-MS, Inductively Coupled Plasma Mass Spectroscopy; LSPR, Localized Surface Plasmon Resonance; MALDI, Matrix Assisted Laser Desorption/Ionization; MPA, Mercaptoacetic acid; NP, Nanoparticle; NPs, Nanoparticles; NTA, Nanoparticle Tracking Analysis; PCS, Photon Correlation Spectroscopy; Pdl, Polydispersity Index; PEG, Polyethylene Glycol; PHEA, Poly(hydroxyethyl Acrylate); PVDF, Polyvinylidene fluoride; SAXS, Small-Angle X-ray Scattering; TiO<sub>2</sub>, Titanium dioxide; UV-Vis, Ultraviolet-Visible; WAXS, Wide-Angle X-ray Scattering; XANES, X-ray Absorption Near Edge Structure; XAS, X-ray Absorption Spectroscopy.

distribution profile, of suspended particles or polymers under Brownian motion. DLS measures the hydrodynamic diameter of nanoparticles, which includes the core particle, its solvation shell, and any adsorbed molecules. The technique is highly sensitive to aggregation, as larger aggregates scatter light more intensely, influencing size distribution results. DLS provides a rapid, non-destructive measurement but is less accurate for polydisperse or non-spherical particles.

The scattering fluctuations are then used to estimate the diffusion coefficient ( $D$ ) applying the Stokes-Einstein equation (Equation 1) to calculate the hydrodynamic diameter ( $d_H$ ) of a particle. This calculation depends on the viscosity of suspending media ( $\eta$ ), the absolute temperature ( $T$ ), and the Boltzmann constant ( $k$ ) as follows:

$$d_H = (kT)/(3\pi\eta D) \quad (1)$$

The obtained diffusion coefficient ( $D$ ) relies on the assumption that particles are spherical in shape and that the viscosity of suspension is known. But, particles in suspension exhibit constant movement and are seldomly spherical. The hydrodynamic particle diameter determined by DLS represents the diffusion of particles in suspension, which means that the obtained diameter provides only an approximate indication of the particle true size. The hydrodynamic diameter, also known as the Stokes diameter, then refers to the size of a hypothetical sphere that has the same translational diffusion coefficient as the particle under analysis (Kastner and Perrie, 2016).

DLS measurement generally yields a distribution dependent on intensity, number or volume. Intensity-based measurements can be used to calculate the number and volume distributions, which are crucial for estimating the relative proportion of particles in a sample (Chu and Liu, 2000). The size distribution is determined by weighing the intensity-based distribution according to the scattered light intensity of each particle. The intensity of scattered light correlates with both the concentration of the analyte and its molecular weight. The absolute molecular weight can be ascertained by integrating light scattering measurements with concentration assessments, such as refractive index measurements. The dispersed light is unaffected by the scattering angle for molecules significantly smaller than the wavelength of the input light (Andersson et al., 2009). DLS is a rapid technique for analyzing particles of very small size, is a highly reproducible technique and with the advantage of not requiring substantial training of the operator. The limitations of DLS are related to its sensitivity to the presence of larger particles or aggregates which can cause intensity measurement distortion (Chu and Liu, 2000; Ito et al., 2004). As a result, this method is not suitable for characterizing particle size distributions that are heterogeneous or multimodal (Bell et al., 2012). Accurate dilution of the sample is also needed to minimize the ratio of signal to noise in the measurements and to prevent the occurrence of multiple light scattering. The quality of filtered medium and clean cuvettes are essential in order to prevent contamination (Kastner and Perrie, 2016).

Sikora et al. (2016) evaluated the size change of silica NPs due to the adsorption of proteins from serum which forms a “protein corona.” The authors reported an increase in particle with the adsorption of proteins at the outer particle layer (Sikora et al.,

2016). However, in case of hydrophilicity and agglomeration, DLS might prove unable to accurately measure the particle size. In such cases, one can rely on other high-resolution techniques such as Differential Centrifugal Sedimentation (DCS).

## 2.2 Differential centrifugal sedimentation (DCS)

Differential Centrifugal Sedimentation (DCS), also known as disk centrifugal photo-sedimentation (DCP) or analytical disc centrifugation, is a novel method for the quantification of NP size distribution as small as 2 nm, and is being used to resolve multi-modal particle distributions and to detect minute variations in particle size (Plüsch et al., 2021). The sample is introduced at the base of a centrifuge chamber, rather than at the fluid interface, enabling particles with a lower density than the fluid to ascend to the fluid’s surface. DCS was used in a study by Kruse et al. (2024), to evaluate the size distribution of ultra-small gold NPs of 1.5 nm covalently conjugated with doxorubicin and a far-red-fluorescent dye. Authors highlighted that the hydrodynamic diameter is routinely underestimated by DCS for extremely small particles due to the unknown effective density of the metal core combined with the hydrated ligand shell (Kruse et al., 2024). Ultra-small metal NPs (mean size of 2 nm), made of silver, platinum, and others, stabilized with glutathione, were characterized by DCS (Wolff et al., 2024b), allowing to confirm the NPs dispersibility in water without signs of agglomeration.

Nakajima et al. (2024) used DCS to assess the adsorptive interactions between ceramic particles and polymeric binders in slurry. Under a centrifugal force, the particles settling induce viscous resistance on the adhered binder, resulting in separation governed by the particle size and density. The authors confirmed that DCS helped in the quantitative evaluation of adsorption forces in the nano-Newton range, translating the interactions between particles and binders (Nakajima et al., 2024).

## 2.3 Nanoparticle tracking analysis (NTA)

In Nanoparticle Tracking Analysis (NTA) technique, the size distribution of particles suspended in a liquid medium is assessed by analyzing their Brownian motion and light scattering (Monakhova et al., 2023). A beam of laser light passing through a prism encounters the suspended particles, which causes scattering of light. This light is focused by an optical microscope onto a camera that captures particle movement, creating a video file. This enables each particle in the frame to be tracked and its specific movement in the suspended medium to be studied (Filipe et al., 2010). It is a comparatively new special technique that can be used particularly for biological systems and molecules, such as DNA and proteins.

As in the case of DLS analysis, diffusion coefficient via NTA can be computed from the distance traveled by the particles, with the help of Stokes-Einstein equation, the distances travelled by each particle in the frame yields real-time information regarding particle size distribution. Besides, the data obtained can be used to display

the number-based distribution, intensity and particle concentration (Kastner and Perrie, 2016).

The smallest particle size that can be estimated by this technique depends on the intensity of the scattered light. The intensity of the scattered light is affected by factors, such as particle size, laser power wavelength, angle and refractive index of the particle and suspending medium, and particle shape. Refractive index of the particles being analyzed determines the lower limit detected. The upper limit, on the other hand, is affected by Brownian movement of the particles which tends to decrease with increase in particle size. NTA measurements are based on the assessment of hydrodynamic diameter of each particle under study. Thus, this avoids errors due to the presence of large particles or their aggregates, being NTA a complementary technique to DLS. NTA usually analyses the motion of individual NPs rather than the sum of their motion. The technique allows higher resolution of sample containing mixed population of particles and analysis of fluorescent particles (Braeckmans et al., 2010). NTA also allows to investigate the size distribution of NPs of diameters in the range of 10–1,000 nm (Filipe et al., 2010). In comparison to DLS, NTA is found to be very accurate for size evaluation of mono- and polydisperse NP populations. NTA, however, requires well-trained personnel for precise measurement. User systematic error in selection of field of view cannot be ruled out. Comparing the time of experiment, NTA usually takes longer time to be carried out than DLS. In addition, the measurement chamber has to be cleaned manually after each measurement (Kastner and Perrie, 2016).

Gross et al. (2016) investigated the particle size, as well as the concentration of variable sized NPs in polymeric suspensions and protein samples. The authors confirmed the very accurate and reproducible particle size measurements and concentration in monodisperse systems, under well-defined and constant parameter conditions (Gross et al., 2016).

Hubert et al. (2020) showed that the NTA technique can potentially be used to characterize submicron-sized protein aggregates and particles in a range of lyophilized and in-solution pharmaceutical products. The authors also showed that the average size of standard 200 nm NPs was within 10% of the expected particle size (Hubert et al., 2020).

## 2.3 Microscopic techniques

Among various imaging techniques available for NPs size analysis, microscopic techniques, such as Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM), represent the most important ones.

### 2.3.1 Scanning electron microscopy (SEM)

Scanning Electron Microscopy (SEM) is a technique used to measure the size of NPs, first detecting their edges. Generally, dimensional metrology of NPs by SEM focuses on particles larger than 10 nm (Feltin et al., 2024). The principle of SEM is the electron scanning, capable of performing resolutions as low as 1 nm, and of providing detailed information regarding the surface morphology of NPs (Khan et al., 2019). SEM can be used to study the morphological characteristics of the nanomaterials, in addition to the NPs dispersion in a matrix or bulk (Khalid et al., 2018).

Wang et al. (2013) described the quadripolar hollow shell structure of  $\text{Co}_3\text{O}_4$  NPs (multi-shelled microspheres) using SEM. These NPs have been reported particularly active as anode in lithium-ion batteries (Wang et al., 2013). The size dispersion and morphology of copper oxide (CuO) NPs have been studied by SEM; Khalid et al. (2018) reported a uniform distribution of the particles and shape of the synthesized oxide (Khalid et al., 2018). SEM was also used to characterize the morphology of silver NPs embedded in chitosan films used as wound dressings (Amaral et al., 2024). Aggregates and irregularities were noted on the material's surface, showing SEM capacity in predicting chemical interactions between silver NPs and the polymer.

In the preparation of the sample for SEM analysis, interactions between the substrate grid and particles, as well as the kinetics of solvent drying, can influence the formation of nanostructures under microscopic analysis. SEM may not be very helpful to analyse sol-gel structures and explain adequately their localized surface plasmon resonance (LSPR) behaviour. This offers some disadvantage of SEM and TEM compared to DLS since this latter does not expose NPs to the risk of clustering, during the preparation of sample due to solvent evaporation (Mourdikoudis et al., 2018).

### 2.3.2 Transmission electron microscopy (TEM)

Transmission Electron Microscopy (TEM) analyzes the crystalline structure and morphology of NPs with high precision (Neumann and Rafaja, 2024). TEM can also provide information about the bulk material, ranging from a too low magnification to a higher one. Further, it provides information about materials having two or more layers (Tomoda et al., 2020). It is one of the most important microscopic techniques that has been employed to characterize systems, such as super-lattice nanocomposites (Shevchenko et al., 2006). It has been reported beneficial for microstructural analysis of biopolymer membranes, including microporous structure developed during film fabrication process (which cannot be observed in SEM). It is also employed to characterize non-reinforcement dispersion in biopolymer composites (Tomoda et al., 2020).

During shelf-life, the structure and characteristics of NPs can change, as a result of their interactions with surroundings (Wiesner et al., 2011). TEM can be used to exploit the interaction taking place between NPs and an electron beam of uniform current density. When the electron beam reaches the particle, it is either scattered or transmitted (Kumar et al., 2019). The magnitude of the resulting interaction is influenced by its size.

TEM has been used to characterize NPs aggregates for numerous biomedical applications. As example, the aggregation of protein A-coated gold NPs in the presence of anti-protein A was monitored by Thanh and Rosenzweig (2002), confirming the aggregation of monodispersed particles as a function of protein concentration (Thanh and Rosenzweig, 2002). Gold NPs were also designed to aggregate in mild acidic intracellular environments by hydrolysis-susceptible citraconic amide surface (Nam J. et al., 2009). The temporal dynamics of the aggregates were analyzed by TEM measurements confirming the electrostatic attractions between gold NPs that have mixed surface charges during the hydrolysis of their surface molecules. However, some limitations are also associated with this technique, such as misleading images because of orientation effects or difficulty in quantification of a large number

of particles. An extra care is needed when preparing the samples so that reliable results are obtained. Improper protocol may lead to erroneous results, e.g., formation of aggregates when the colloidal suspension is subject to drying.

An advantage of TEM is that this technique helps to assess the alteration in subcellular structures caused by NPs (Blanco-Andujar et al., 2016). DLS and TEM have been used to check the polymeric coating biodegradation in NPs, which may give rise to colloidal aggregation, hampering their mobility and causing cytotoxicity (Kirschling et al., 2011). Interaction of NPs and cells is commonly studied combining TEM with confocal laser scanning microscopy (CLSM) (Ostrowski et al., 2015). Frequently, these two techniques are used together, since CLSM permits live imaging of cells and fluorescent labelling of various components of cell, while TEM gives higher resolutions than other imaging techniques (Cutler et al., 2012). Hence, TEM frequently employed to determine the located NPs within a cell (Yue et al., 2017).

TEM was used to assess the size and shape needed to ensure high cellular uptake and vesicle escape of gold NPs from vesicles (Yue et al., 2017).

Hussain et al. (2020) synthesized gold NPs using different polarity indices of ethanol-water mixtures and carried out TEM imaging. The resulting images revealed nearly spherical NPs measuring 9.7 nm and 13.9 nm produced, respectively, by 20% and 50% volumetric percentage of ethanol to water. TEM images of gold NPs prepared using 80% volumetric percentage of ethanolic solution displayed however irregularly shaped and larger sized particles (approximately 53.1 nm), which were present as aggregates in the colloidal dispersion.

TEM helped to disclose the mechanism of cell death resulted from Magnetic field hyperthermia treatment of melanoma cells with iron oxide NPs, which led to the formation of vacuoles as a result of apoptosis (Blanco-Andujar et al., 2016).

### 2.3.2.1 High resolution transmission electron microscopy (HR-TEM)

High Resolution Transmission Electron Microscopy (HR-TEM) is based on the principle of phase contrast imaging, in which scattered and transmitted electrons create an image. This technique provides very high resolution and allows the detection of atoms in crystalline structures. Hence, HR-TEM is one of the most commonly used techniques to conduct characterization of internal structure of NPs (Mourdikoudis et al., 2018). However, if inner structure of NPs attain random orientation relative to the source of electrons, there may be regions where the atoms are misaligned, resulting in complex images (José-Yacamán et al., 2001; Khan et al., 2020). HR-TEM was found useful in studying the palladium nanoclusters (with size falling between 1 and 1.5 nm) prepared by colloidal methods (José-Yacamán et al., 2001). The study by José-Yacamán et al. (2001) revealed that the Pd mono constructions were a mixture of four differently shapes of structured crystals (FCC cubo-octahedral, icosahedral, decahedral and twinned), being those structures with comparable energy levels (José-Yacamán et al., 2001). The use of HR-TEM has been reported for the study of effect of ligands in the structure of platinum NPs, produced by organometallic synthesis (Axet et al., 2011).

Khan et al. (2020) combined two techniques, viz. bright-field TEM (BF-TEM) and phase-contrast HR-TEM, to record information about particle size, shape and crystallographic structure of silver NPs. The images were derived from films deposited on copper TEM grids. The accelerating voltage was set to a value of 300 keV, while the distance between the sample and the detector was 11.8 mm. The layer of silver NPs on the surface exhibited a clear morphology and narrow size distribution, with a mean size of approximately 8 nm. When compared to SEM, a smaller particle size obtained by TEM was due to the smaller sample deposit on TEM grid, positioned 20 mm farther from the ablation point. Phase contrast imaging of the crystal lattice displayed fringe spacing, suggesting crystalline structure of silver NPs (Khan et al., 2020).

HR-TEM was also used to evaluate the degree to which NPs penetrate to different tissues (Gontier et al., 2008). Sunscreens containing TiO<sub>2</sub> NPs of size range of 20–100 nm were tested on several skin samples (human skin grafted on immuno-deficient mice models, healthy human skin and porcine skin), with HR-TEM showing that the particles were retained in the upper corneocyte layers of the *stratum corneum*.

SEM and TEM provide high-resolution imaging of nanoparticles, offering structural and morphological insights. However, both techniques generate 2D projections, which may not fully represent the actual 3D shape of nanoparticles. TEM is particularly effective for high-resolution imaging at the atomic scale but requires careful sample preparation to minimize beam damage.

### 2.3.2.2 Cryo-Transmission electron microscopy (Cryo-TEM)

Cryo-Transmission Electron Microscopy (cryo-TEM) allows the near-unaltered samples to be visualized in their frozen-native environment by freezing them to cryogenic temperatures, using liquid nitrogen. Cryo-TEM calls for tedious sample preparation, but it enables direct imaging of the NPs in their native environment (Tkachenko et al., 2020).

Tkachenko et al. (2020) used cryo-TEM for the characterization of core-shell spherical NPs falling in sub-50 nm range prepared using poly(hydroxyethyl acrylate)-b-poly(styrene) (PHEA23-b-PS130) by polymerization-induced self-assembly method (Tkachenko et al., 2020). The average diameter of prepared NPs was 37.0 ± 5.5 nm. A close examination of sample derived at high magnification showed the existence of dark spots within the particles. These were ascribed to the presence of extended PHEA chains oriented parallel to the direction of electron beam (Tkachenko et al., 2020).

### 2.3.3 Atomic force microscopy (AFM)

Atomic Force Microscopy (AFM) is another microscopic technique employed to generate 3-D images of the surface, size and morphology of the sample at higher magnification. This technique is used in a wide range of fields, such as for the characterization of biological systems, materials and in physics, with the advantages of simple sample preparation and of high-resolution topography measurement. What limits its monitoring in relation to dynamic processes and subsequent applications is the low scanning speed of traditional AFM (Xu et al., 2022; Mulholland et al., 2024).

AFM is based upon measurement of interacting forces between the sample surface and a fine probe. The probe consists of a sharp tip (in the nm order) made of silicon nitride or silicon, which is coupled to one end of a cantilever. When AFM scans the sample, due to repulsive and attractive forces (between the sample surface and the probe), the cantilever gets deflected. A laser beam is present to quantify the deformation, which is reflected on the backside of the cantilever. The forces are computed by consolidating the data from non-cantilever stiffness and laser variation. AFM scans under three variable modes on the basis of the degree of proximity between the sample and probe (tapping, contact and non-contact mode) (Vilalta-Clemente et al., 2008; Hameed et al., 2018). The tapping mode is the most commonly used for characterization of NPs. AFM can be used for imaging any type of surface. Samples which exhibit poor contrast in electron microscopy can be characterized by AFM. Furthermore, when compared to electron microscopy (TEM and SEM), AFM is capable of providing comparable results for size analysis of NPs (Vilalta-Clemente et al., 2008). It also allows the evaluation of NPs' height. It requires a small floor space, displays laser scanning times and provides comparable resolutions to TEM and SEM. It provides results similar to DLS, when sample is uniform and monodisperse (Oćwieja et al., 2013).

Hosseingholilou et al. (2020) prepared thin films of gold NPs and results of AFM surface analysis indicated that these thin films had an appropriate uniformity and similarity (Hosseingholilou et al., 2020). Khan et al. (2020) employed AFM to characterize films of silver NPs for their topography (2D and 3D surface landscapes). On average, over 20 surface line profiles were found in the region. The surface line profiles exhibited a variation of the order of 3–5 nm, confirming the presence of fine features. The mean particle size was 40 (22) nm, as derived from the distribution histogram. The value showed slight variation from SEM observation, which could be attributed to tip limitation and some other artifacts that are a part of AFM measurements. In addition, a duplicate convoluted image was obtained with better contrast and reduced noise. This image enabled the discrimination of smaller features present on the surface (Khan et al., 2020).

### 2.3.4 Confocal laser scanning microscopy (CLSM)

Confocal Laser Scanning Microscopy (CLSM) comprises an epifluorescence microscope equipped with multiple laser sources and a confocal scan head that includes fluorescent filter sets, a galvanometer-based raster scanning system, multiple or adjustable pinhole apertures, and photomultiplier tube detectors for diverse wavelength ranges (Hard et al., 2014).

CLSM is commonly used for the tracking of NPs in biological media. Ruseska et al. (2025) used this technique for the elucidation of the cell uptake of nanostructured lipid carriers (NLC) bearing miRNA in two distinct cell lines (3T3-L1 and MCF-7). For the imaging of the cells, the actin cytoskeleton was stained with Alexa Fluor™ 488 Phalloidin, and the nuclei was stained with the blue-fluorescent DNA stain DAPI (4',6-diamidino-2-phenylindole). The fluorescently labelled Cy3-NLC were seen to be taken up and internalized by the 2 cell lines (Ruseska et al., 2025).

Curcumin-loaded lyotropic liquid crystal NPs were characterized by CLSM to evaluate the three-dimensional formation of the lamellar structure (de Souza et al., 2020). It

could be seen that, under low hydration or dried state, lipids adopted the typical crystalline lamellar phase.

## 2.4 X-ray diffraction (XRD)

X-Ray Diffraction (XRD) represents one of the primary techniques to describe the structural properties and crystallinity of NPs. This method provides a rough estimation regarding particle size, using Debye Scherrer formula (Khan et al., 2017; Kumar and Rao, 2019), besides allowing the identification of single and multiple NPs (Emery et al., 2016). However, for NPs with sizes lower than 100 of atoms, the proper measurement and acquisition of structural attributes may pose problems. Besides, the diffractograms can be influenced by NPs with amorphous properties and variable atom-atom bonds (Ingham, 2015).

X-ray diffraction is known to vary according to the atoms composing the crystal lattice and their arrangement. When a sample is exposed to a beam of X-ray and undergoes diffraction, the distances between the planes of atoms can be measured employing the Bragg's Law (Equation 2):

$$n\lambda = 2d \sin \theta \quad (2)$$

where  $n$  is an integer in the order of the diffracted beam,  $\lambda$  represents the wavelength of the incident beam of X-ray,  $d$  denotes the distance between adjacent planes of atoms (the  $d$ -spacings), and  $\theta$  is the angle formed by incidence of the X-ray beam. The  $d$ -spacings can also be calculated as the geometry of the XRD instrument is specifically designed to facilitate this measurement. The sample's particular set of  $d$ -spacings is what allows the X-ray scan to provide a precise "fingerprint" of the material. XRD can also estimate crystallite size using Scherrer's equation, making it a key tool in nanoparticle characterization. The interpretation of the scan entails comparison with the standard references patterns, on the basis of which the materials are identified (Pandian, 2014).

Statistically representative data are among the benefits of XRD. However, the diffractogram obtained for NPs with size less than 3 nm may be too broad. Moreover, the samples meant for XRD analysis need to be in powder form, which is usually obtained by drying the colloidal dispersion of NPs (Mourdikoudis et al., 2018), and this treatment can modify the polymorphism of the particles under study.

Upadhyay et al. (2016) checked the size of magnetized Fe<sub>3</sub>O<sub>4</sub> particles by means of XRD with the Scherer formula using the broadening of the most intense peak. The size of Fe<sub>3</sub>O<sub>4</sub> particles ranged from 9 nm to 14 nm when the synthesis temperature increased from room temperature to 100°C. The size increased from 27 nm to 53 nm with increasing the reaction period from 10 min to 2 h. The lattice parameter for the analyzed samples ranged from 0.8397 to 0.8414 nm (Upadhyay et al., 2016).

Nath et al. (2020) published a study on XRD peak broadening analysis by different models. Cadmium selenide (CdSe) NPs were prepared by first reducing selenium powder into sodium hydrogen selenide (NaHSe) with sodium borohydride (Nath et al., 2020). To this selenium precursor, a mixture of distilled water, 3-mercaptopoacetic acid and cadmium chloride was added to finally obtain CdSe NPs. XRD was used for the analysis of their structural

attributes and crystallinity. XRD peak broadening analysis was carried out to estimate the elastic properties of the CdSe NPs. Various properties studied with the help of Scherrer plot were stress, intrinsic strain and energy density. Models, such as Williamson–Hall plot, Halder-Wagner Method and Size-Strain Plot were employed, in addition to particle size determination. The values of average particle size of CdSe NPs determined by TEM and SEM analysis were found to be 36.23 nm and 36.2 nm, respectively. The authors confirmed that the values obtained from Halder-Wagner Method and Size Strain Plot for the particle size of CdSe NPs were close to those obtained from TEM, SEM and AFM analysis (Nath et al., 2020).

Similar to XRD technique, small-angle X-ray scattering (SAXS) method allows elastic scattering at a specific angle in a solid; however, the SAXS detector covers small angle of scattering (Modrow, 2005). Typically, this technique is used to measure the size, size distribution and shape of NPs.

Wang et al. (2008) utilized SAXS to assess the structural changes in platinum NPs as a function of temperature. Wide-angle X-ray scattering (WAXS) allows the diffraction maxima at larger angles because the distance between the detector and the NPs sample is smaller than in SAXS (Wang et al., 2008).

Navarro Oliva et al. (2024) used both SAXS and WAXS in the size characterization of nanocomposites based on Fe<sub>3</sub>O<sub>4</sub> and polyvinylidene fluoride (PVDF) of 6, 9, and 14 nm. SAXS and WAXS allowed to distinguish  $\alpha$ -phase into the  $\beta$ -phase of PVDF (Navarro Oliva et al., 2024). The scattering patterns yielded information regarding the size and structure of the NP aggregates, the lamellar structure of the PVDF (Navarro Oliva et al., 2024), and besides informing the type of  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  polymorphs (Concha et al., 2024).

## 2.5 X-Ray absorption spectroscopy (XAS)

X-Ray Absorption Spectroscopy (XAS) ascertains the X-ray absorption of the sample as a function of energy. Every element has an exquisite set of characteristic absorption edges which correspond to different binding energies of its electrons, enabling element selectivity by XAS. This technique encompasses X-ray absorption near edge structure (XANES or NEXAFS) and extended X-ray absorption fine structure (EXAFS) methods (Mourdikoudis et al., 2018). It provides information regarding coordination number, bond distances and disorders of the neighboring atoms. As it does not need any long-range order, it can be widely employed for a variety of materials with some modifications in mechanical and optical design, entire XAS spectrum can be recorded with millisecond resolution. Further, it is one of the most appropriate tools to observe structural modification during a reaction process and the change in the oxidation state of metal cations *in situ*. Despite these advantages, one major challenge of all X-ray based techniques is that they yield ordinary information. Therefore, XAS can be complementary to other techniques, such as XRD, UV-visible spectroscopy, Fourier Transformed Infra-Red (FTIR), Raman and mass spectroscopy, for broader view of the NPs and more consolidated results (Nayak et al., 2018). Wu et al. (2002) (Wu et al., 2002) employed XAS for detailed structural determination of titanium oxide NPs. de Oliveira et al.

(2020) coupled XAS with X-ray diffraction and used these techniques to explore the bottom-up mechano-synthesis of gold NPs in the presence of different reducing agents. The authors demonstrated that these techniques provide new directions for mechanochemical research of advanced electronic substances (de Oliveira et al., 2020).

## 2.6 UV-visible spectroscopy (UV-Vis)

Ultraviolet visible (UV-Vis) spectroscopy is another low-cost characterization technique often employed for investigation of nanoscale materials (Carpentieri and Domenici, 2024). It is capable of handling a great number of NPs and the sample preparation is relatively easy (Tomoda et al., 2020). In this technique, intensity of light reflected from a sample is measured and compared to the intensity of reflected light from the reference standard (Tomaszewska et al., 2013). NPs possess optical characteristics, and their shape, concentration, size, state of agglomeration and refractive index are other features which make UV-Vis spectroscopy a vital technique to carry out for identification, characterization and study of NPs stability (Mourdikoudis et al., 2018). Sensitivity, simplicity and selectivity of this technique, together with the short duration of measurement and no requirement of calibration, are some merits of UV-Vis spectroscopy over other size determination techniques (Tomaszewska et al., 2013). This technique is also a non-destructive robust tool for quick recognition of the different types of NPs. Silver, copper and gold NPs exhibit typical UV-visible extinction spectra in the visible region. The absorbance (350–400 nm) can also be employed to determine the gold colloid concentration (Mourdikoudis et al., 2018).

UV-Vis spectroscopy was used to confirm the synthesis of silver NPs from fungal isolates (Santos et al., 2022) and from hydroalcoholic extract of leaves of *Schinus terebinthifolius* Raddi (de Oliveira et al., 2021). Haiss et al. (2007) (Haiss et al., 2007) published an interesting study on the use of UV-Vis spectra to assess the concentration and size of gold NPs, varying between 5 and 100 nm. Hussain et al. (2020) (Hussain et al., 2020) used UV-Vis spectroscopy to characterize gold NPs in varying ethanol-water volumetric ratios in the solvent mixture. The maximum surface plasmon resonance (SPR) absorption wavelength depends on the size and shape of NPs. The maximum wavelength value of the prepared gold NPs exhibited a shift, meaning that the size of NPs increased with the increase of ethanol-water volumetric ratio. The maximum wavelength of gold NPs produced in a solvent mixture with 20% and 50% volumetric percentage of ethanol was found to appear in the shorter absorption wavelength regions (514 nm and 520 nm), which suggested that gold NPs synthesized with lower ethanol percentage were smaller in size. Also, the maximum wavelength of NPs, prepared with ethanol-water volume percentage of 80%, displayed a shift towards higher wavelength regions. This implies that larger sized particles were formed, while a broad trend of the plots indicates unevenly shaped gold NPs (Hussain et al., 2020).

TABLE 1 Examples of techniques commonly used for the characterization of nanoparticles and purposes.

Technique	Size range (nm)	Information detected	Sample preparation	Applications
AFM	0.5 to 1000	NP shape and size (in 3D mode), dispersion of NPs in cells and matrices, lateral dimensions of NPs, quick analysis of elemental composition	Samples must be rigid, adhere to a substrate and disperse on substrate. Substrate must be appropriately chosen	Nanostructure visualization and measurement at nanoscale for surface roughness and step-height
DCS	2 nm to 50 $\mu$ m, depending on particle density	NP size and size distribution	Sample should be delivered to bottom of the centrifuge chamber, rather than to the surface of the fluid	Analysis of ceramic oxides, emulsions, fillers, polymer latexes and abrasives (of all types), pigments, inkjet inks, oil emulsions, microspheres, liposomes
DLS	10 to 1000	Hydrodynamic size, detection of agglomerates, intensity, number and volume-based size distribution	10–500 $\mu$ L (concentration dependent)	Investigation of wider range of dispersions, colloids and suspensions
FMR	-	NP shape, size, size distribution, surface composition, crystallographic imperfection M values, demagnetization field, magnetic anisotropic constant	Geometry of the sample is significant	Assessment of magnetic characteristics of ferromagnetic materials, applied to a wide range of materials from nano-scale magnetic thin films to bulk ferromagnetic materials
ICP-MS	1–100 nm	Composition and concentration of nanoparticles	Sample preparation is required prior to ICP-MS evaluation to quantify the total concentration of nanoparticle suspensions	Elemental composition, and number, concentration in a single, rapid analysis. Determine the concentration of dissolved analyte in the sample
MALDI	-	Chemical information (surface-sensitive) on functional group, surface topography, molecular orientation and conformation MALDI for NP size	Optimization of the sample preparation as per the physical and chemical properties of analytes is important. The samples should be appropriately sealed to avoid drying during storage	Analysis of trace quantities of substances in biological samples due to their large surface area for analyte enrichment, efficient desorption and homogeneous sample crystallization
NMR	-	Electronic core structure, ligand density and arrangement, atomic composition, influence of ligands on NP size and NP shape	NMR samples should be prepared in NMR tubes (5 mm)	Protein-nanoparticle interaction measurement, nanoparticle size measurement
NTA	10 to 2000	Sizing, size distribution, particle concentration	Sample should be in suspension form; Wide range of solvent can be used. Sample size- 500 $\mu$ L; Temperature- 5°C–50°C	Assessment of colloids, suspensions, synthetic and biological particles
SEM	0.1 to 1000	High-resolution imaging of nanoparticles, offering structural and morphological insights(2D images)	Samples should be either sputter coated or conductive	Evaluation of morphological, topographical and compositional parameters
TEM	0.1 to 1000	High-resolution imaging of nanoparticles, offering structural and morphological insights(2D images), NP shape, mean size and polydispersity, aggregation state, homogeneity, lattice size	Sample size < 1 $\mu$ g, and needs to be prepared as a thin film which is stable under high vacuum and electron beam	Detect and quantify NPs in matrices, morphological information, crystallographic details, composition of phases
Cryo-TEM	0.1 to 1000	Structure of materials in liquids in nm resolution in two and three dimensions, with subsecond time resolution	Sample preparation requires skilled handling and careful technique through a number of detailed preparation steps	Helps to study aggregation pathways, complex growth mechanisms, good for colloid chemistry and molecular biology to avoid sample destroying and presence of artefacts
HR-TEM	0.1 to 1000	Structure, structural defects, structural changes, surface profile imaging	Samples should be transparent to the electron beam	Provides all information similar to conventional TEM but also regarding crystal structure of single particle. Distinguish between amorphous, monocrystalline and polycrystalline NPs
XAS	1 to 1000	X-ray absorption coefficient (element-specific), chemical state of species Debye-Waller factors, interatomic distances, for crystalline as well as non-crystalline NPs	Wide variety of sample types in different environmental conditions	Provides local structural parameters at the atomic level
XRD	1 to 1000	Average particle size, crystal structure, composition, crystalline grain size	Larger crystalline samples (>1 mg) required	Characterization of materials, determination of particles in suspensions

(Continued on following page)

TABLE 1 (Continued) Examples of techniques commonly used for the characterization of nanoparticles and purposes.

Technique	Size range (nm)	Information detected	Sample preparation	Applications
				or polycrystalline solids, study of phase transitions in a substance, semi-quantitative analysis of phases in a sample, measurement of sample size in nanomaterials
UV-visible spectroscopy	-	Optical properties, size, concentration, agglomeration state, hints on NP shape	Filled the cuvette approximately $\frac{3}{4}$ full, with given sample	Detection of impurities and structural elucidation of organic compounds. Unknown substances can be identified. The concentration of known substances can be determined. Measure the absorbance of the blank at one wavelength, or over a wavelength range

Abbreviations: AFM, Atomic Force Microscopy; DCS, Differential Centrifugal Sedimentation; DLS, Dynamic Light Scattering; FMR, Ferromagnetic resonance; ICP-MS, Inductively Coupled Plasma Mass Spectrometry; MALDI, Matrix Assisted Laser Desorption/Ionization; NMR, Nuclear Magnetic Resonance; NTA, Nanoparticle Tracking Analysis; SEM, Scanning Electron Microscopy; TEM, Transmission Electron Microscopy; Cryo-TEM, Transmission Electron Cryo-microscopy; HR-TEM, High-resolution Transmission Electron Microscopy; XAS, X-Ray Absorption Spectroscopy; XRD, X-Ray Diffraction; UV, Ultraviolet.

## 2.7 Nuclear magnetic resonance (NMR) spectroscopy

Nuclear Magnetic Resonance (NMR) Spectroscopy is a useful analytical method for structural and quantitative characterization of nanomaterials. NMR was used to study interaction between ligand and surface of magnetic NPs (Wolff et al., 2024a). This technique helps in the routine investigation of molecular scale NPs, in both solid and solution phases. Particularly, it is useful for analysis of the production and final concentration of metallic NPs (Mourdikoudis et al., 2018). NMR also provides a more convenient and advanced way of surface chemistry evaluations and size determination of NPs. All NMR reports have inherent low sensitivity of Zeeman splitting (Marbella and Millstone, 2015). Guo and Yarger (2018) employed NMR spectroscopy for thoroughly studying characterization of gold NPs for surface chemistry investigation, size determination and structural evaluations. The sizes of gold NPs assessed by NMR agreed with TEM outcomes (Guo and Yarger, 2018).

Other useful techniques for NP size measurement include ferromagnetic resonance (FMR), dynamic light scattering (DLS) or photon correlation spectroscopy (PCS), and matrix assisted laser desorption/ionization (MALDI). Inductively coupled plasma mass spectroscopy (ICP-MS) is a powerful elemental analysis technique used to determine the composition and concentration of nanoparticles. While it does not directly measure nanoparticle size, single-particle ICP-MS (spICP-MS) can estimate size distributions by analyzing individual nanoparticle ionization events.

Table 1 lists examples of size measurement techniques applied to NPs for different size ranges and purposes.

## 3 Effect of mean size on the toxicological profile of nanoparticles

Unique properties of NPs, such as shape, surface characteristics and size, need to be optimized in order to capitalize their potential for different biomedical applications (Khan et al., 2019). Thus, the risk of toxicity arising due to their nano size is largely overlooked (Yang et al., 2017). Due to their small size, NPs can be a source of

toxicity, both when used in medical applications and when released into the environment. NPs toxicological characterization aims to elucidate the interaction between the physicochemical features of these NPs and their biological targets. Hence, thorough understanding of the interaction taking place between biological systems and NPs is needed. Studies correlating the size of NPs with toxicity using cells in cultures, as well as animal models, are being undertaken. These studies in particular, aim to assess the effect of size of NPs on their *in vitro* and *in vivo* toxicological profile, paving the way towards the fabrication of appropriate carriers for various therapeutic applications (Abbasi et al., 2023; Qamar et al., 2024).

### 3.1 *In vitro* toxicity evaluation of nanoparticles

The merits of *in vitro* toxicity assessment include relatively lower cost and fewer ethical concerns, when compared to the *in vivo* screening assays. *In vitro* cytotoxicity is mainly assessed through necrosis, apoptosis, proliferation, oxidative stress and DNA damage assays (Lama et al., 2020; Tirumala et al., 2021). Evaluation of *in vitro* toxicity using various cell lines has been used and reported in a number of publications [e.g., (Silva et al., 2019a; Silva et al., 2019b)]. Cell viability and lethality are the two most common characteristics assessed for *in vitro* measurement of toxicity for different types of NPs (Silva et al., 2019a; Silva et al., 2019b; Mao et al., 2022).

An increased interest in investigating the effects of NPs size on targeting cellular uptake has been observed over the years (Augustine et al., 2020; Galindo-Camacho et al., 2023). Numerous research groups have conducted *in vitro* studies involving variable cell types and different NPs, and assessed the impact of size and surface charge on their uptake into the cells and on drug targeting.

#### 3.1.1 Cellular uptake studies

Cellular processes in mammals, such as endocytosis, cellular uptake and inflammatory response, have been found to be closely related to particle size (Augustine et al., 2020). Most common mechanisms of NPs cytotoxicity are listed in Table 2.

TABLE 2 Common cytotoxicity mechanisms promoted by nanoparticles. Summary of main cell targets and the putative mechanism of toxicity.

Cellular target	Mechanism of action
Lipids and proteins	Formation of reactive oxygen species (ROS), free radical cascades may result in cell components oxidation
Cell membrane	Cell membranes structure and integrity may be compromised/disrupted, leading to altered permeability, oxidative stress, ion imbalance, and cell death
DNA	ROS or NPs-induced DNA single- and double-strand breaks, disturbing transcription leading genetic mutations, or epigenetic modifications, influencing long-term cellular responses
Mitochondria	NPs can disrupt mitochondria function, inducing oxidative stress, changes in mitochondrial potential and permeability, resulting in cell energy imbalance, cytochrome c release and apoptosis
Lysosomes	Interfere with lysosome targeting, hampering autophagy and macromolecules degradation and consequently triggering apoptosis
Membrane proteins	Cause structural changes to membrane proteins, disturbing their function, such as transport of materials in and out of cells, catalysis etc.
Immune response	Trigger the synthesis of inflammatory mediators via the release of cell components from damaged cells. Interaction with cell defense mechanisms
Cytoskeleton	Interrupt extracellular/intracellular signaling, altering or damaging cytoskeleton architecture, preventing intracellular transport, cell division and leading to apoptosis

The NPs size determines their interactions with biological systems (Augustine et al., 2020). Although a great deal of work has been carried out to investigate the toxicological effects of NPs in a systematic manner based on their size, the field still demands scientific attention so that the internal fate of the nanosized materials may be disclosed and their toxic potential may be ascertained. This is especially important in case where studies of similar nature performed by different research groups bring out contrasting outcomes.

For a given geometric shape and composition, NPs dimensions are a determining factor of cell uptake (Augustine et al., 2020). NP size influences the entropic and enthalpic features, which impact their cellular uptake by governing the adhesion forces between the cellular receptors and NPs (Villanueva-Flores et al., 2020). Hoshyar et al. (2016) (Hoshyar et al., 2016) reported that most *in vitro* investigations have shown the highest cellular uptake of NPs with size ranging between 10–60 nm, regardless their composition and surface charge (Hoshyar et al., 2016). In case of materials with the same chemical composition but varying size, numerous physical characteristics and chemistries may be anticipated. NPs have large surface area, therefore, the particle surface is of paramount importance as a large number of atoms are located at the surface (Gnach et al., 2015). It has been reported that NPs with size falling below 5 nm are able to circumvent cell barriers by non-specific means (e.g., translocation). On the other hand, particles with larger size access the cells via phagocytosis, macro pinocytosis, and specific and nonspecific transport mechanisms. Pinocytosis, however, depends on cell type and size of particles, since a size of around 25 nm is optimal (Manzanares and Ceña, 2020; Sousa de Almeida et al., 2021).

For cellular uptake, NPs undergo membrane wrapping process via endocytosis of ligand coated NPs and this mechanism of action is size dependent (Li et al., 2022). NPs of size above 60 nm in diameter may lead to a large interaction with a number of receptors limiting the binding of other NPs in a competitive fashion. NPs with diameters below 30 nm attach to some receptors on the

membrane surface but are unable to drive the membrane-wrapping process. NPs ranging between 30 and 60 nm may bind to membrane receptors, usefully driving the membrane-wrapping process.

Pan et al. (2007) studied the size dependent toxicity of gold NPs, sized in the range 0.8–15 nm. The study was conducted on fibroblasts, macrophages, epithelial cells and on melanoma cells, revealing that particles measuring 15 nm were around 60 times less toxic than those measuring 1.4 nm in size (Pan et al., 2007). The authors also found that 1.4 nm sized NPs led to cell necrosis (within a period of 12 h subsequent to the culture medium) (Pan et al., 2007).

In a study carried out by Jiang J. et al (2008) (Jiang W. et al., 2008) using gold NPs, it was reported that the particles measuring 40–50 nm displayed the highest cellular uptake in SKBR-3 cells (Jiang W. et al., 2008). The same results were obtained when the core of NPs was changed to silver (Jiang W. et al., 2008). Studies carried out with HeLa cells using mesoporous silica NPs showed a maximum cellular uptake for particles with 50-nm size (Lu et al., 2009). Zhang et al. (2009) (Zhang et al., 2009) reported the development of a thermodynamic model that exhibited optimum uptake within the cells when the diameter of a ligand-coated NPs was 50 nm (Zhang et al., 2009).

In a study by Huo et al. (2014) (Huo et al., 2014), it was found that gold NPs not greater than 6 nm effectively gained entry within the cell nucleus, while larger sized NPs, i.e., measuring 10 or 16 nm, only crossed the cell membrane and could be found only in the cytoplasm. These findings are particularly interesting in case ultrasmall gold NPs are aimed for gene therapy, having the DNA as their target (Huo et al., 2014).

Braakhuis et al. (2016) evaluated the cytotoxicity of inhaled silver NPs of different sizes. Although the primary determinant of whether NPs reach the alveoli and where they can induce toxicity was found to be their size, the authors also reported the need for a dose metric to assess the risk of nanomaterials of the same chemical composition but varying in size (Braakhuis et al., 2016). This study

also described a concentration-dependent rise in lung toxicity markers, i.e., cell counts and pro-inflammatory cytokines, in the bronchoalveolar lavage fluid (Braakhuis et al., 2016).

Carnovale et al. (2019) performed a systematic evaluation of the impact of shape, size and surface capping by developing a set of gold NPs, on cytotoxic behavior and cellular uptake. A reduction in the size of gold NPs resulted in an enhanced cellular uptake, in terms of particle number (Carnovale et al., 2019). Additionally, gold NPs of the same size, stabilized using amino acids, displayed elevated cellular uptake levels, in comparison to citrate or cetyltrimethylammonium bromide (CTAB) coated gold NPs (Carnovale et al., 2019).

Lee et al. (2019) evaluated the cytotoxic effects of silica NPs on human endothelial cells. Unlike other size ranges, silica NPs of ca. 20 nm notably induced both necrosis and apoptosis; however, these two mechanisms occurred independently and via different pathways (Lee et al., 2019). Reactive oxygen species-mediated endoplasmic reticulum (ROS-mediated ER) stress was responsible for apoptotic cell death. Autophagy-mediated necrotic cell death was caused via pI3K/AKT/eNOS signaling axis (Lee et al., 2019).

Chakraborty et al. (2020) investigated the size dependent biological activity of gold NPs on osteosarcoma cells. Gold NPs of size range of 40–60 nm were synthesized by a Tris-assisted citrate-based method, whereas surface enhanced Raman scattering (SERS) was used to confirm size dependent apoptosis of osteosarcoma cells treated with these particles (Chakraborty et al., 2020).

### 3.1.2 Hemolytic activity

*In vitro* assays, particularly those assessing hemolytic activity, offer an efficient approach for preliminary toxicity screening; however, these methods often overlook key aspects, such as the influence of plasma proteins and mechanical stress on NP-hemocompatibility. NP toxicity tests should consider disease-specific red blood cells, such as diabetes and hemoglobinopathies (Yedgar et al., 2022). Additionally, the effect of NP size on the hemolytic activity of cells is also worth pondering upon. A study carried out with 100–600 nm mesoporous silica NPs on human erythrocytes established that a large specific surface area of NPs leads to their effective adsorption on cell surface. Particles measuring 100 nm in size were better adsorbed on the surface of human erythrocytes without leading to any alteration in the cell morphology, whereas particles measuring 600 nm entered the cells, causing membrane deformation and hemolysis (Sukhanova et al., 2018).

### 3.1.3 Administration routes

NPs have become a part of daily life of humans, where they may find access to the human body either in the form of pharmaceutical products, meant for therapeutic or diagnostic purpose, or as a constituent of cosmetic products or accidentally in the form of environmental pollutants (Gnach et al., 2015). Human exposure to nanoparticulate matter may occur via occupational exposure through inhalation and/or ingestion, but also through any drug administration route (Kumah et al., 2023). Investigations in this regard have correlated organ toxicity with pre-determined dose. However, exposure to NPs may have some non-dose dependent effects as well. Since the reduction of the particle size increases the surface area per unit volume, particles become more chemically reactive with the size reduction (Jiang J. et al., 2008).

The extent of toxicity is influenced by the route of administration, as well as by the sites of NP deposition (Lara et al., 2011). Depending upon the route of entry of the NPs, different cell lines have been employed for their *in vitro* testing. For example, for oral uptake of NPs, cell lines such as Caco-2, HT29, and SW480 have been used (Andreani et al., 2014; Silva et al., 2019a). When intended for intravascular route, HeLa, MCF-7, BT-474, PC3, C4-2, and SKBR-3 have been employed (Doktorovova and Silva, 2018; Souto et al., 2019). To study the transdermal route, keratinocytes (such as HaCaT cells (Carbone et al., 2019b)), fibroblasts, and, rarely, sebocytes, have been proposed, while for inhalation uptake RAW 264.7, BEAS-2B, NHBE, 16-HBE, SAEC, and THP-1 cell lines have been used (Sukhanova et al., 2018). Conventional cell-based assays designed to evaluate chemicals may however yield unreliable and erroneous results when employed for the testing of nanosized materials. This is probably because NPs tend to interact with the read-out systems and assay components.

A great deal of research has already provided extensive information and a variety of data; however, a clear understanding of the results is still limited due to the absence of standardized assessment procedures and inconsistencies in the model employed, media used, reference standards adopted and analysis of collected data. Kroll et al. (2012) suggested that classical cytotoxicity assays need to be designed, so that they can be validated for every NP type, and the testing concentration of the NPs must be limited to levels below which interference is likely to occur (Kroll et al., 2012). Data comparison regarding SLN *in vitro* cytotoxicity (using various cell models), oxidative stress and hemocompatibility studies also revealed the inexistence of standard protocols and data analysis tools which may lead to contradictory results (Doktorovova et al., 2014b; Zielińska et al., 2020).

## 3.2 *In vivo* toxicity evaluation

*In vivo* toxicity evaluation brings NPs a step closer to its clinical relevance. Precision in the control of physicochemical characteristics, such as size, surface charge, shape and stability of NPs, is of utmost importance in *in vivo* studies since these properties govern their residence time in the blood stream and also their site specific targeting properties (Kumar et al., 2017; Sabourian et al., 2020; Yagublu et al., 2022). *In vivo* toxicological evaluation is generally performed using appropriate animal models. The choice of an adequate experimental model for estimating toxicity (*in vitro* and *in vivo*) is of vital significance. *In vitro* models (cell lines) easily help to analyze toxic effects of nanoparticulate matter on individual components of cells and tissues, but *in vivo* evaluation makes it possible to assess the NPs toxicity for individual organ and to the whole organism (Ajadary et al., 2018; Gnach et al., 2015). The assessment techniques for *in vivo* toxicity comprise studies regarding biodistribution, clearance, hematology, histopathology and serum chemistry.

The most commonly employed animal models, for *in vivo* studies, include mice and rats, due to their explicit genome, which makes them most appropriate for studying the histopathological changes and functions of various organs (such as hepatic, hematological, renal, nervous and immune systems).

Besides these, rabbits, zebra fish, *Drosophila melanogaster* and *Caenorhabditis elegans* also find use in toxicological investigations (Yang et al., 2017; Severino et al., 2023).

*In vivo* assessment studies may be focused on changes in the tissue structure (Jans and Huo, 2012), apoptosis (Mallidi et al., 2009; Sharma et al., 2009), inflammation and infiltration in major organs (spleen, kidney, brain, lungs and heart), but may also be aimed to investigate certain organ systems, which tend to concentrate NPs on account of their structures specificity (Yang et al., 2017). Elucidating the biodistribution of NPs in various organs may prove beneficial to guide the optimization of NPs, in all respects including their size. After animal sacrifice, the main organs or tissue are removed, commonly to explore distribution of NPs, but also to assess morphological and histological changes and signs of toxicity. The ultra-structural modification and effect of the NPs on tissue can be clearly revealed employing electron microscopy analyses. Functional injury can be detected with the help of diverse biomarkers and sophisticated high-tech instruments (Yang et al., 2017; Joseph et al., 2023). Furthermore, NPs can be detected in live or sacrificed animal models through radio labelling (Dai et al., 2021).

Gold NPs were found to cause apoptosis and acute inflammation in liver, accumulation in various organs, as well as penetration potential in sperm head and tail regions (Cho et al., 2009; Wiwanitkit et al., 2009). A study was carried out with the aim of checking the *in vivo* toxicity of zinc NPs at concentration of 14–20 µg/mL for 12 h in order to ascertain the impact of treatment on DNA damage, cell viability, apoptosis and ROS production (Sharma et al., 2012).

Silver NPs biodistribution and toxicity were investigated using CD-1 mice by intravenous injection of 10 mg/kg of silver NPs with different particle size, i.e. 10, 40, and 100 nm, possessing different shell coatings. Each type of synthesized NPs caused toxicity to tissues, but larger sized particles were less toxic, apparently because they are less prone to penetration (Recordati et al., 2015).

Jimeno-Romero et al. (2016) evaluated the biodistribution, bioaccumulation and biological effects of disodium laureth sulfosuccinate (DSLS) stabilized titanium dioxide (TiO<sub>2</sub>) NPs in marine mussels (*Mytilus galloprovincialis*). This *in vivo* study used 0.1, 1, and 10 mg titanium/L concentration, either in bulk or as TiO<sub>2</sub> NPs (sized 60 and 180 nm). A considerable accumulation of NPs was noted in mussels dosed with TiO<sub>2</sub> NPs, localized in lysosomes, endosomes and residual bodies of digestive cells, as well as in the lumen of digestive tubules. The NPs measuring 60 nm were internalized to a larger extent in lysosomes of digestive cells as compared to 180 nm ones. This was confirmed by quantitative analysis of black silver deposits following autometallography (Jimeno-Romero et al., 2016). The larger sized particles were found in the lumen of gut where they were present as large aggregates. This resulted in notably decreased stability of lysosomal membrane to both the types of NPs, though greater in extent upon exposure to those with 60 nm diameter. The membrane stability was also affected by exposure to TiO<sub>2</sub> in bulk and DSLS-stabilized NPs. Therefore, it was concluded that membrane stability was dependent on the size of the NPs, and also on the biological effects caused by DSLS and TiO<sub>2</sub> (Jimeno-Romero et al., 2016).

Balmuri et al. (2017), Balmuri et al. (2017) synthesized two different types of cadmium oxide NPs, by calcinations of Cd(OH)<sub>2</sub> without any organic molecule (CdO-1) and with cadmium-citrate

coordination polymer (CdO-2). The authors compared the toxicity of the two types of cadmium oxide NPs, on zebra fish showing those obtained by calcination with CdO-2 reduced toxicity, which was revealed by lower levels of oxidative stress, rescue of hepatic carboxylesterases and diminished metallothionein activity. These results were also supported by histopathological examination. The study articulated the toxic effects of cadmium oxide NPs in aquatic animals and also illustrated that the toxicity could be significantly reduced by carbon coverage (Balmuri et al., 2017).

Kang et al. (2020) designed infrared fluorophore conjugated polyethylene glycols (PEGs) in different sizes, and investigated their size-dependent enhanced permeability and retention (EPR) effect, and checked their pharmacokinetics, biodistribution and renal clearance in tumor-bearing mice. The targeting efficiency of PEGylated particles was confirmed by measuring tumor-to-background ratio. While the smaller sized variants of PEGs (≤20 kDa, 12 nm) displayed marked tumor targeting with either very low or non-specific uptakes, the larger sized variants (>20 kDa, 13 nm) were found to accumulate significantly in major organs, such as liver, lungs and pancreas (Kang et al., 2020).

### 3.2.1 Genotoxicity evaluation

Genotoxicity can appear directly at DNA, as well as at other chromosome components (Doktorovova et al., 2014a; Doktorovova et al., 2014b; Souto et al., 2020; Cardoso et al., 2021a). Modifications in the genetic material can result in serious health issues in humans and can be responsible for chronic diseases (Kohl et al., 2020). Genotoxicity agents affect DNA and non-DNA targets of cells via several modes of action. Poorly soluble particles have strong tendency to produce ROS, which are responsible for their genotoxicity (Oberdörster et al., 2005; Schins and Knaapen, 2007). Because of their high surface reactivity when compared to microparticles, NPs can produce high ROS levels when compared to larger sized particles (Knaapen et al., 2004; Schins and Knaapen, 2007; Gonzalez et al., 2008).

The potential risk of nanoscale particles in causing genetic damage is assessed through genotoxicity testing. This involves selecting a series of particles made of the same material, but with progressively smaller sizes, to evaluate the impact of size on DNA damage. Gel electrophoresis assay or comet assay are then used to quantify DNA single strand breaks in cells (Cardoso et al., 2021b; Menz et al., 2023). DNA damage results in gene mutation, altered protein synthesis, followed by altered mechanisms of cell cycle arrest which may lead to carcinogenesis (Singh et al., 2017).

The research conducted by Barnes et al. (2008) focused on the examination of genotoxicity by single cell gel electrophoresis or Comet assay performed on a single fibroblast cell line (3T3-L1) to examine five distinct commercial colloidal and laboratory-synthesized silica NPs. None of the tested NPs exhibited any notable genotoxicity. Remarkably, these findings were confirmed independently in two distinct research facilities, demonstrating that *in vitro* genotoxicity testing may be accurately replicated across different laboratories (Barnes et al., 2008). Nevertheless, caution is needed in the interpretation of the findings that indicate that amorphous silica NPs are not hazardous (Fubini et al., 2010).

Exposure conditions, such as dose and duration, are also significant in determining toxicity of NPs. In addition to size, shape, and the level of hydrophilicity/hydrophobicity, the

TABLE 3 Selected studies evaluating *in vitro* and *in vivo* toxicity of NPs.

Type of nanoparticles	Particle size (nm)	Toxicity evaluation				References
		<i>In vivo</i>		<i>In vitro</i>		
		Organs	Animals	Cells	Cell line	
Gold NPs	10 and 50	Liver and kidney	Wistar Kyoto rats			Abdelhalim and Moussa (2013)
Gold NPs	20–115			Human prostate cancer cells	PC3 cells	Carnovale et al. (2019)
Gold NPs	5–35 (different shapes)			Cancerous and healthy cells	U87 and human dermal fibroblast cells	Bhamidipati and Fabris (2017)
Gold NPs	86	Embryos	Zebrafish			Browning et al. (2013)
Gold NPs	36, 46 and 60			Osteosarcoma cells	MG63	Chakraborty et al. (2020)
Gold NPs	5–15			Mouse fibroblast	Balb/3T3 cells	Coradeghini et al. (2013)
Gold NPs	1.5–13.6			Human embryonic stem cell	hESC cells	Senut et al. (2016)
Gold NPs	1.5, 4.5, 13, 30 and 70	Heart, lung, liver, kidney	Mice			Yang et al. (2009)
Citrate coated gold NPs	10, 11, 25			Human epithelial cells	HDMEC and hCMEC/D3 cells	Freese et al. (2012)
Colloidal gold NPs	20–200				DU-145	Vedantam et al. (2013)
PEG-coated gold NPs	5, 10, 30, 60	Bone marrow, blood cells, liver and spleen	Mice			Zhang et al. (2011)
Fluorophore-conjugated PEG NPs	2–16	Targeting all organs	Mice			Kang et al. (2020)
Silver NPs	60, 85			Human carcinoma cell lines (human lung and human colon carcinoma cell lines)	A549 and HCT 116	Dasgupta et al. (2016)
Citrate coated silver NPs	10, 40 and 75, also 10 (coated), 50 (uncoated)			Human lung cells	BEAS- 2B	Gluga et al. (2014)
Silver NPs	10–80			Mammalian cells	Mammalian fibroblast	Ivask et al. (2014)
Silver NPs	20, 50 and 75			Pulmonary epithelial cells	16HBE140	Kettler et al. (2016)
Silver NPs	10, 50, 100			Mouse prosteoblast, Rat adrenal medulla derived, human cervical cancer cells, Chinese hamster ovaries cells	MC3T3-E1, PC.L2, HeLa and CHO	Kim et al. (2012)
Silver NPs	4, 20, 70			Liver cells	U937	Park et al. (2011)
Citrate coated silver NPs	20 and 110	Gill and intestine	Zebrafish			Osborne et al. (2015)
Citrate coated silver NPs	20, 60, 100		<i>Daphnia magna</i>			Seitz et al. (2015)
Iron oxide NPs				Mammalian fibroblast line	Balb/C373 and COS-1	Harris et al. (2015)
Iron oxide NPs	5 and 30			Porcine aortae	Porcine aortic endothelial cells	Yu et al. (2012)
Silica NPs	50 and 200			Neuronal cells	GT1-7	Ariano et al. (2011)

(Continued on following page)

TABLE 3 (Continued) Selected studies evaluating *in vitro* and *in vivo* toxicity of NPs.

Type of nanoparticles	Particle size (nm)	Toxicity evaluation				References
		<i>In vivo</i>		<i>In vitro</i>		
		Organs	Animals	Cells	Cell line	
Silica and dendritic NPs	3–165	Tissue, blood, organ (heart, lungs, liver, spleen, kidney, GI tract)	Mice and Sprague Dawley rats			Greish et al. (2012)
Gold-PLLA-, PCL-coated silica NPs	60 and 80			Neuronal cells, microglial cells, (undifferentiated and differentiated)	SH-5Y5Y AND N9	Koch et al. (2014)
Silica NPs	20, 30, 40 and 50			Human umbilical cord vein	HUVECS	Lee et al. (2019)
Silica NPs	20 and 100			Kidney cells	Human HK-2 and porcine LLC-PK	Passagne et al. (2012)
Silica NPs	32 and 83			Colon epithelial cells	CaCo-2	Schubbe et al. (2012)
Tungsten carbide cobalt NPs	98			Lung epithelial cells	BEAS-2B	Armstead et al. (2014)
Cadmium NPs	Nanospheres- 50 and 80, nanowires- Length- 400-500 Width- 20-30		Zebrafish			Balmuri et al. (2017)
Co-polymer NPs	45–90			Rat macrophages and human adenocarcinoma derived	NR8383 and CaCo2	Bhattacharjee et al. (2012)
TiO <sub>2</sub> NPs	14–196			Neonatal rat calvaria, human embryonic kidney and normal liver cells	Osteoblasts, HEK293, L-02	Cai et al. (2011)
TiO <sub>2</sub> NPs	60 and 180	Digestive gland	Mussels			Jimeno-Romero et al. (2016)
Hydrogenated castor oil NPs	920, 454 and 151		Kunming mice		BHK-21	Chen et al. (2014)
Selenium NPs	35 and 91		Kunming mice	Human squamous cell carcinoma	Tca8113	Wang et al. (2015)

regulation of particle absorption also significantly influences genotoxicity (Prior et al., 2002; Nam H. Y. et al., 2009). Research on the toxicity of nano-sized particles has demonstrated that these are more harmful than micrometer-sized particles of the same composition. This finding has prompted concerns about the potential influence of NPs on human health (Carbone et al., 2019a). For example, copper oxide NPs have significantly higher toxicity in comparison to copper oxide micrometer-sized particles. On the other hand, the small particles of TiO<sub>2</sub> generated a greater amount of DNA damage compared to TiO<sub>2</sub> NPs, most likely due to differences in their crystal structures. While the iron oxide NPs did not exhibit toxicity that varied with size, their toxicity was comparatively lower than that of other NPs (Gautam and van Veggel, 2013). Although significant advancements have been made, accurately predicting the toxicity behaviour of NPs remains challenging due to the diverse range of concentrations, sizes, shapes, surface coatings, exposure times, and other factors involved in *in vitro* and *in vivo* experiments. This complexity makes it highly difficult to anticipate the toxicity of NPs for a new formulation. Table 3 lists examples of studies reporting the *in vitro* and *in vivo* toxicity assessment of NPs.

## 4 Conclusion

Nanotechnology has offered exciting new alternatives for therapeutics and diagnostics in the past 2 decades attributed to the range of new types of nanoparticles (NPs) that were developed and physicochemical characterized using a number of analytical techniques. NPs are classified within the size range varying from 1 to 100 nm. Various types of NPs including gold NPs, citrate-coated gold NPs, colloidal gold NPs, PEG-coated gold NPs, polymeric NPs, silver NPs, citrate coated silver NPs, iron oxide NPs, silica NPs, gold-PLLA-coated silica NPs, PCL-coated silica NPs, tungsten carbide cobalt NPs, cadmium NPs, TiO<sub>2</sub> NPs, hydrogenated castor oil NPs, and selenium NPs, have been discussed for their versatile application. Though these fabricated NPs have been a subject of extensive research, and are known for their utility, still several questions remain unanswered and ethical concerns surround their use. Their fabrication is based on the assumption that they are formulated using biodegradable and biocompatible ingredients. Studies on their action at the cellular and systemic levels, allowing for the generation of correlations between the physicochemical properties of NPs and biological responses, are still needed to

ultimately serve as a blueprint for the development of future NPs. Computer simulation studies can also play an important role in designing NPs for a specific application. Further, to address the limited data available about safety and toxicity of NPs, it is important to incorporate toxicity research from the early stages of formulation development towards the generation of safer formulations, based on the comprehensive assessment of each NP-based formulation to establish complete toxicity safety profiles. This will help meet the regulatory agency requirements and expedite the approval process.

## Author contributions

WSM: Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft. PS: Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft. VK: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft. RR: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft. AZ: Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft. AMS: Supervision, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – review and editing. SM: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft. EBS: Conceptualization, Supervision, Data curation, Formal Analysis,

Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – original draft.

## Funding

The author(s) declare that financial support was received for the research and/or publication of this article. Eliana B. Souto acknowledges University College Dublin for the Research Scheme fund 2024–2028 (82934-NP/R27885). Amélia M. Silva acknowledges FCT–Portuguese Foundation for Science and Technology, for the support of the project UID/04033:CITAB.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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