Review

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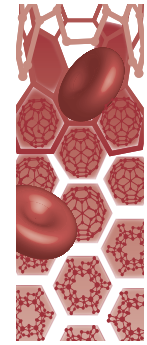
Nanomedicine



Nanotoxicology: advances and pitfalls in research methodology

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As research progresses, nanoparticles (NPs) are becoming increasingly promising tools for medical diagnostics and therapeutics. Despite this rise, their potential risks to human health, together with environmental issues, has led to increasing concerns regarding their use. As such, a comprehensive understanding of the interactions that occur at the nano-bio interface is required in order to design safe, reliable and efficient NPs for biomedical applications. To this end, extensive studies have been dedicated to probing the factors that define various properties of the nano-bio interface. However, the literature remains unclear and contains conflicting reports on cytotoxicity and biological fates, even for seemingly identical NPs. This uncertainty reveals that we frequently fail to identify and control relevant parameters that unambiguously and reproducibly determine the toxicity of nanoparticles, both *in vitro* and *in vivo*. An effective understanding of the toxicological impact of NPs requires the consideration of relevant factors, including the temperature of the target tissue, plasma gradient, cell shape, interfacial effects and personalized protein corona. In this review, we discuss the factors that play a critical role in nano-bio interface processes and nanotoxicity. A proper combinatorial assessment of these factors substantially changes our insight into the cytotoxicity, distribution and biological fate of NPs.

Keywords: cellular uptake • nano–bio interface • nanomedicine • nanoparticles • nanotoxicology • organ-on-a-chip • preanalytical factors • toxicity

Nanoparticles (NPs) have emerged as promising candidates for various diagnostic and therapeutic applications [1–4], such as targeted drug delivery [5,6], targeted (molecular) imaging [7], contrast agents [8], gene transfection [9] and hyperthermia [10]. Meanwhile concerns over the health and environmental impacts of NPs are also on the rise, leading to efforts with a goal of understanding the potential short- and long-term toxicity of NPs [4,11]. The ways in which cells sense, respond to, and are potentially damaged by NPs is numerous [12]. NP-induced changes can be intracellular (membrane disruption, DNA damage, mitochondria malfunction, production of reactive oxygen species [ROS], genotoxicity and the activation of apoptosis pathways) [13] or extracellular (e.g., leading to the release of inflammatory cytokines) [14]. Furthermore, NP-induced dysfunctional changes may go beyond cellular changes and involve structures such as the blood brain barrier [15], tissue organization and the integrity of organs.

The physicochemical properties of NPs (their size [16,17], shape [17], surface charge [18], hydrophilicity/hydrophobicity [19] and surface roughness [12]) have important effects on their interactions with biological systems, but our current understanding of these effects has provided us with limited predictive power [20–28]. In addition to the physicochemical properties of NPs, their environmental parameters (e.g., the composition of biological fluids), alongside the nature of the target cells significantly affect the biological fate of NPs. The combinatorial nature of assessing toxicity based on NP characteristics,

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environmental factors and target cell properties, makes it very difficult to determine the health risks associated with NP exposure in a reliable and reproducible fashion. Many of the *in vitro* nanotoxicity assays currently used are ones that have been developed for molecular toxicology. However, the limitation of these assays is that the behavior of NPs differs dramatically from that of molecular toxicants, thus complicating their use for nanotoxicology. For example, one major methodical challenge specific to nanotoxicology, not accounted for by current *in vitro* assays, is NP interference with assay components and/or detection systems. Moreover, in designing molecular toxicity assays, we do not generally account for parameters that are relevant to NPs, such as: protein corona, aggregation status, cell shape and exposure conditions; all of which strongly affect the toxicity and physiological responses generated by NPs [29–35]. These issues diminish the reproducibility of current *in vitro* nanotoxicity assays and more importantly, the ability to predict *in vivo* results [36]; an outcome that is corroborated by many conflicting reports about the toxicity of these materials [37,38]. In the literature, several NPs that were previously thought to be safe were later shown to have direct and indirect toxic effects *in vivo* [14]. Conversely, toxic effects ascribed to NPs were later found to arise from other factors such as ions or solvents in solution [39,40].

There are a number of methods that specifically probe the toxic effects of NPs (Figure 1); but these methods are by no means comprehensive. Many of the disagreements between the available nanotoxicology reports arise from studies overlooking one or several parameters that influence the toxic impact of NPs [12,24]. For example, because of NP's ability to affect cell viability through various intra- and intercellular pathways, a reliable assessment must take into account an NP's effect on all relevant pathways. Some pathways that should be assessed include: the induction of apoptosis, the activation of immune responses, the generation of reactive oxygen species (ROS) [41], cell membrane disruption and genotoxicity (Figure 2) [42–46]. Another important factor, which is usually not thoroughly addressed in nanotoxicology assays, is the use of 'standardized controls'; used to ensure that experimental observations are real occurrences, rather than artifacts. Some examples of these controls, such as the need for a supernatant control, are addressed in a later section of this review.

This review will primarily focus on recently discovered factors, with demonstrated effects on the toxicity of NPs. Furthermore, state-of-the-art nanotoxicity assays will be discussed; with a close look at their reliability, alongside the effects of NP interference on the assays. Ultimately the need for new standardized methods, which consider proven critical assessment factors, will be highlighted. This need will be demonstrated by looking at sources of disagreement in the current literature, followed by proposed solutions to alleviate these discrepancies.

Important factors in nanotoxicity assays

Characterization of NP physicochemical properties

The physicochemical properties of NPs, including their size, shape, charge, functionality, stability, solubility, chemical composition, surface modification, agglomeration and sedimentation states, play a crucial role in their toxicity and biological responses [47,48]. As a result, the effective and consistent characterization and reporting of NP physicochemical properties is essential in the field of nanotoxicology [49,50]. In (patho)physiological environments, the behavior of particles can be quite complex and much different from what we see in routine laboratory settings [47]. To this end, the properties of NPs must be quantified under (patho)physiological conditions, so that the results can be translated to toxicity studies. Another challenge that often arises is routed in inconsistencies between various measurement methods. For example, different particle sizing techniques will produce different results and, as with any method, the physical principle, physical property being measured, algorithm used, basis of the distribution and dynamic range of the instrument will not be the same. As a result, specifications quantified based on the use of dynamic light scattering or other techniques, such as Brunauer–Emmett–Teller or transmission electron microscopy, should not be considered comparable. An example of these variations was seen in a study by Powers *et al.* [51] which showed that as-received aluminum NPs with a nominal size of 30 nm can have variations in size ranging from 34 microns to 31 nm, depending on the measuring technique. Therefore, to conduct an accurate particle size analysis, one should ideally have a representative sample, a well-dispersed system, and a physical measurement technique (or combination of different complementary techniques) that is carefully selected to produce data that is relevant for the intended use. When in doubt, it is often wise to combine the results of multiple techniques, or to refer to standards such as International Organization for Standardization or American Society for Testing and Materials in order to conform to common practice [51].

The precise and accurate characterization of NPs is further complicated due to the fact that, unlike bulk materials, NPs have a high tendency to agglomerate, especially in wet states. Factors such as the shear (e.g., mixing or sonication), fluid characteristics (e.g., pH, zeta potential) and NP concentration

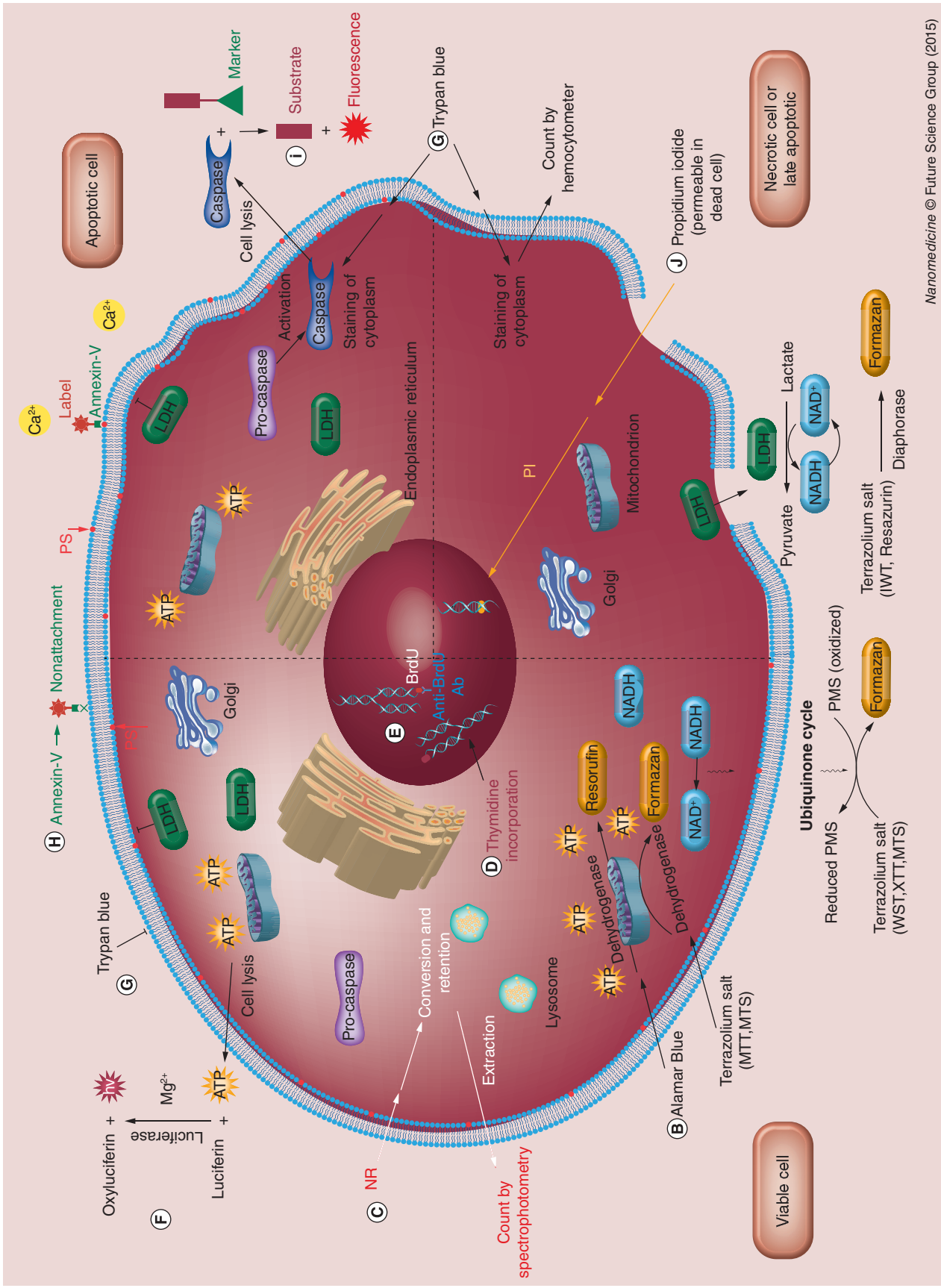

Figure 1. Mechanisms of action for common nanoparticle toxicity assays. **(A)** MTT viability assay, **(B)** Alamar Blue viability assay, **(C)** neutral red uptake assay, **(D)** thymidine incorporation assay, **(E)** BrdU staining, **(F)** luciferase assay, **(G)** Trypan blue dye exclusion assay, **(H)** flow cytometry-based Annexin V apoptosis assay, **(I)** caspase–3 fluorescence apoptosis assay and **(J)** flow cytometry-based propidium iodide staining for viability and DNA content analyses. These methods are classified on the basis of their abilities to detect apoptotic cells, late apoptotic cells, necrotic cells and reductions in cell viability.
For color figures, please see online at www.futuremedicine.com/doi/full/10.2217/NNM.15.130

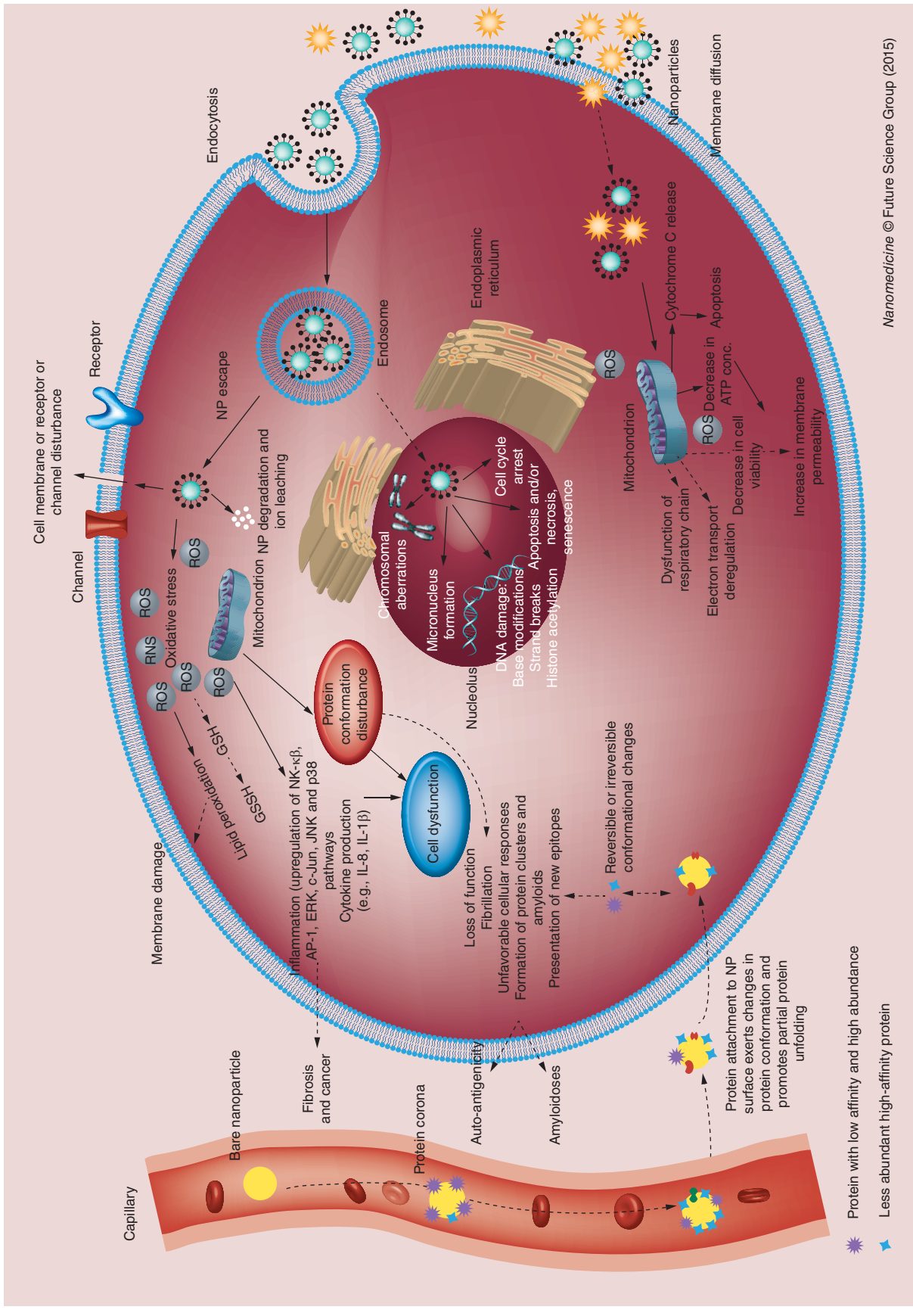

Figure 2. Mechanisms of nanoparticle-induced cell death. NPs affect cell viability via different mechanisms including the disruption of cell membranes, exerting conformational changes in the attached protein corona, lipid peroxidation, saturation of cellular defense pathways, induction of inflammation and/or immune responses, production of ROS and oxidative stress, ion leaching (through NP degradation), exertion of unfavorable signaling pathways, interference with mitochondrial activity, disturbance of cell receptor and/or channel activity, formation of micronuclei, cell cycle arrest, DNA damage, and chromosomal aberrations leading to apoptosis, necrosis or senescence.
NP: Nanoparticle; ROS: Reactive oxygen species.

can influence the NP agglomeration process. Coccini *et al.* [52] demonstrated that agglomeration is a key factor in determining the cytotoxicity of carbon nanotubes. Surface properties, such as hydrophobicity and charge, also influence the toxicity and cellular uptake of NPs; primarily because of the variation in the colloidal stability of the NPs [53]. Considering the aforementioned factors, multiple characterization techniques are required to reduce inconsistencies between studies [17]. Full details on the importance of NP physicochemical characterization and its effects on toxicity evaluations have been presented in previously conducted reviews [47–50,54–57].

Purity of NP solutions: the need for a supernatant control

For pharmaceutical products in clinical use, toxicity or undesired side effects can be exerted by an active pharmaceutical ingredient (API) itself or by residual components that are associated with chemical synthesis; such components include solvents, crosslinkers and surfactants. For all approved pharmaceuticals, there are specification limits in place for these impurities in order to prevent toxic effects on the patient, upon administration of the drug. For example, an exposure level of 1.5 µg per person, per day, for each impurity can be considered an acceptable qualification threshold to support a marketing application, provided that the impurities are not known to be carcinogenic or genotoxic compounds [58]. This threshold exposure level for compounds does not imply a significant risk for carcinogenicity or other toxic effects. Similarly, an NP solution contains residual chemicals/impurities from synthesis that may contribute to a toxic cellular or *in vivo* response to the solution. Unfortunately, this fact is rarely considered in the design of nanotoxicological evaluations. For example, Alkilany *et al.* showed that the toxicity of a cationic gold nanorod solution originally arises from the presence of free cationic quaternary ammonium surfactants that were used in its synthesis (namely, cetyltrimethyl ammonium bromide, or CTAB) [59]. Infact, the supernatant of a CTAB-GNP solution had a similar toxicity profile to that of the 'parent' GNP solution prior to centrifugation. Moreover, researchers found that cationic CTAB-GNPs are not cationic in cellular media because of the spontaneous formation of an anionic protein corona. With this in mind, the researchers provided support against the generally held belief that cationic NPs are more toxic than their anionic counterparts due to their ability to disrupt negatively charged membranes through electrostatic means. Instead, the toxicity of cationic nanoparticles may arise from the presence of 'cationic free surfactants/molecules' in the solution rather than the cationic NPs themselves [60]. The presence of these free surfactants/molecules is a significant problem in an 'impure NP solution'; a valid issue due to fact that a thorough cleaning of an NP solution is difficult without resulting in an irreversible aggregation, as CTAB-GNPs aggregate after three centrifugation cycles.

NP dosages: therapeutic vs effective doses

The cellular uptake and toxicity of NPs are correlated with the available NP concentration, which is in many cases lower than the therapeutic dosage, as it can be affected by various factors between the time of administration and arrival at the site of action. NP toxicity can arise through both direct contact with biological compartments, and the release of toxic molecules or ions upon particle exposure. Differentiating between these two types of cytotoxicity is difficult because of the dynamic and complex nature of both dose-dependent mechanisms [61–63]. In most studies, the NP dose is reported as mass per unit volume (e.g., µg/ml), however the dose of NPs that is delivered to adherent cells on the culture plate might not reflect the realistic dose. Cho *et al.* showed that factors generally overlooked by researchers, such as diffusion velocities or sedimentation, may in fact be the primary determinants of cellular uptake of Gold NPs which influence the aforementioned changes in concentration [64]. Reporting the exposure dosage, rather than the nominal concentration, provides a better description of the administered NP concentration [65].

Conventional methods of measuring cellular uptake are not accurate enough to allow for an estimation of the available NP concentration. An example of such errors can be observed in reports in which the uptake of gold nanoparticles (GNP) by adherent cells is monitored by measuring the decrease in their plasmon absorbance in cell culture medium. These data are commonly reported without considering the nonspecific adsorption (loss) of GNPs into culture plates or as aggregates. Despite the ability of a variety of more sensitive analytical tools, such as mass spectrometry (MS), inductively coupled plasma MS (ICP-MS), and liquid chromatography MS (LC-MS), to determine the NP contents of cells, relatively few studies employ these techniques to make an accurate determination of NP contents in cells [65]. Another source of error in estimating cellular uptake is the commonly held assumption that all cell-associated NPs are internalized. This scenario is typically observed when destructive analytical techniques, such as ICP-MS, in which the differentiation of internalized NPs from surface-adsorbed NPs is practically impossible, are employed [66]. Therefore, difficulties in determining cellular exposure to NPs

are compounded by a lack of quantitative knowledge about cellular uptake.

Correlating *in vitro* and *in vivo* dosages presents an additional challenge. Ideally, the NP dosage that is employed for *in vitro* toxicity should be representative of the NP quantity that is present within *in vivo* systems [67]. Current *in vitro* assays are often unrealistic models of *in vivo* systems, with the employed *in vitro* NP dose being completely different than the dose at the target sites of *in vivo* models [68]. For example, Donaldson *et al.* [67] showed that there is no correlation between the toxic (inflammatory) NP dose, as measured *in vitro* based on the induction of inflammatory phenotypes in lung epithelial cells, and the *in vivo* threshold concentration for initiating inflammation in the proximal alveolar region of the lungs.

In vitro modeling of target cellular systems

Co-cultures & 3D cell cultures

Tissues and organs are typically made up of multiple cell types embedded in an extracellular matrix. Depending on what subsets of cells are incorporated into the *in vitro* assay, along with the presence or absence of a matrix, the outcomes of the *in vitro* toxicity assays vary [48,65]. The presence of more than one cell type in a culture medium can alter the toxicity behavior of NPs; the presence of different cell types, and an extracellular matrix, may influence the accessibility of NPs to the target cell, and consequently affect the susceptibility and the response of the cell as well. Furthermore, the mixed effect of an alteration in biological pathways, such as the expression of proinflammatory mediators or stress-related genes, can also influence the results [43].

Dimensionality is a second concern with *in vitro* models. The vast majority of cytotoxicity evaluations in NPs are performed in 2D cell culture environments [36,43,69,70]. However, tissues and organs are 3D, and thus 2D cell cultures are not fully representative of the 3D *in vivo* cellular environment [71]. Common 2D cell cultures do not replicate numerous cell-cell and cell-matrix interactions. For example, in granular epithelial cells, the enhanced specific protein-secreting function can only be observed when the cells form a 3D-organized acinus structure [72]. In addition, the diffusion and transport of NPs in 2D systems differs markedly from those seen in 3D *in vivo* conditions [73,74].

New 3D culture models have been developed to reduce the inherent differences between 2D *in vitro* plated cell cultures and *in vivo* systems [73]. For example, Lee *et al.* [74] demonstrated that the toxic effects of CdTe and gold NPs are substantially higher in 2D culture media than in 3D-spheroid-culture models. They also concluded that phenotype alteration and tissue-like morphology are critical factors that cause the observed enhancement in the biocompatibility of gold NPs in 3D systems. Consistent with these findings, Movie *et al.* showed that single-walled carbon nanotubes (SWCNTs) increase the expression of interleukin IL-6 and tumor necrosis factor TNF-α, and significantly reduce the viability of cells that were cultured in a 2D medium; however, they have little or no effect on cell viability and the expression of inflammatory cytokines in a 3D cell culture model [75]. It can thus be concluded that 3D models have a greater capability to replicate *in vivo* conditions.

Protein coronas

The NPs that are in contact with physiological media are covered by proteins, giving the individual particles completely different surface properties. This adsorption layer, the 'protein corona', alters the surface properties of NPs and hence dictates their behavior in biomedical applications [76]. The composition of the protein corona is strongly dependent on the physicochemical properties of NPs, and the conditions present in the physiological media, such as the temperature, protein source and media composition [77,78]. The effect of the corona on small NPs (<10 nm) is particularly strong; allowing them to enter the cells via pathways that are distinct from phagocytosis or pinocytosis [77,78]. The biomolecules in the corona may be recognized by cell membrane receptors and cause regulated uptake, thus affecting their internalization rate. The formation of a protein corona has been shown to affect NP toxicity and biological activities such as cellular uptake and targeting capabilities [79,80]. For example, Lesniak *et al.* [81] demonstrated that, in a serum-free medium, silica NPs engaged in tighter adhesion to the cell membrane and thus resulted in higher cellular uptake efficiency, as opposed to a medium which contained serum, where looser adhesion resulted in less efficient uptake. The authors then went on to show that the formation of a protein corona also affected the localization of NPs in cells and their consequent toxicity. In another study, Lesniak *et al.* suggested that the primary reason for low cellular adhesion and the uptake of NPs with protein coronas may be related to the reduction of nonspecific interactions between NPs and cells [82]. Similarly, Hu *et al.* demonstrated that protein coronas could reduce the cytotoxicity of graphene sheets, as they weaken or prevent the direct interaction between graphene oxide and cells [83].

The biological effects of corona-coated NPs are partly related to the conformation of proteins in the corona. For example, the structural changes in fibrinogen that are induced by gold NPs trigger an immune

response via the activation of the Mac-1 receptor [14]. Furthermore, Prapainop *et al.* [84] demonstrated that the CdSe/ZnS-metabolite (cholesterol 5,6-secosterol atheronal-B), which induces unfolding in the apolipoprotein B corona, results in the specific binding of the unfolded protein to distinct macrophage receptors; leading to high cell-specific-uptake efficacy. The conformation of the protein corona also governs the interaction of corona-coated NPs with receptors on nonimmune cells. For example, it was found that, due to the induced conformation changes, bovine serum albumin (BSA) molecules that adsorbed to anionic NPs bound specifically to albumin receptors, while BSA molecules adsorbed on cationic NPs were found primarily bound to scavenger receptors [85]. Another example of the effect of conformation changes is that transferrin, an iron-binding blood plasma glycoprotein, irreversibly loses its native conformation and function when exposed to superparamagnetic iron oxide NPs [86]. Recently, Monopoli *et al.* [87] introduced protein glycosylation/modification as a novel factor, at the nano-bio interface, that regulates protein corona formation and subsequently modulates NP-cell interactions, cell uptake and inflammation. In addition to the aforementioned examples, Dawson *et al.* have also conducted a study in protein organization and epitope expression on the NP surface. They showed that the assembly of proteins on the NPs is random, and that a small fraction of bound proteins present functional motifs [88]. The protein conformation of the corona and the motif exposure on the NP surface are key parameters that should be considered when studying NP-protein interactions. Therefore, it can be concluded that the toxic/therapeutic impacts of NPs are, in part, related to the protein conformation of the corona. Future direction, based on this conclusion, should lead to thorough investigation of the protein corona conformation and extensive epitope mapping, in an effort to fully understand the actual biological impact of NPs.

Temperature

Despite extensive research on the role of NP physicochemical properties in cellular interactions and protein corona compositions, there are few reports about the effects of body temperature on NP behavior [89]. Different parts of the human body have different basal temperatures, varying over a range of approximately 3°C [90]. Moreover, in cases of cancer, inflammation or infection, the organ/site temperature has the potential to increase past its normal range [91]. Temperature variations can significantly affect the composition of proteins in the NP corona [35]. For example, the binding of human serum albumin (HSA) and apotransferrin proteins to NPs decreases significantly with increasing incubation temperature [35]. In fact, even slight changes in temperature affected the protein corona composition and consequently influenced the cytotoxicity and cellular uptake of NPs [92].

Interestingly, the local heating induced by either the plasmonic heating of metallic NPs or the magnetic heating of hyperthermic NPs results in considerable changes in the protein corona composition, distinct from conventional heating [34]. Local heating induces protein conformational changes and consequently affects protein replacement on the NP surface. These studies indicate that the tight regulation of the temperature within a physiologically relevant range is required for nanotoxicity assays to be meaningful.

Plasma concentration & protein sources

Plasma concentration is the primary factor for determining the protein corona composition. Monopoli *et al.* showed that, for polystyrene NPs, the association of 50–70-kDa proteins within the corona composition was enhanced by increasing the protein concentration [93]. The composition of the protein corona also changed when the NPs (made from polystyrene and silica) were incubated in different plasma concentration gradients. In a recent study, Ghavami *et al.* showed that the composition of a hard corona (consisting of tightly bound proteins that are not readily desorbed), that formed on the surface of polystyrene and silica NPs, depended on the plasma protein concentration; in particular for low molecular weight proteins [94]. This finding implies that the composition of the protein corona will change dramatically during NP transport to different parts of the body because of the differences in composition of the media in the various parts of the body [95,96] .

Another important factor that determines the composition of the corona is the source of medium used for the assay, such as human plasma, human serum, or fetal bovine serum. These biofluids differ significantly in content and they can form protein coronas with differing compositions and thicknesses on the surfaces of NPs [97]. For instance, the effects of different protein sources (fetal bovine serum and human plasma) on the composition and thickness of the hard corona that formed at the surface of superparamagnetic nanoparticles, with various sizes and surface chemistries, was probed [97]. The results of this analysis, showing variations in the composition of the respective coronas, supported the fact that the properties of the hard coronas were considerably dependent on the protein source.

'Personalized' protein coronas

Patients can react very differently to identical active pharmaceutical agents (API) [98,99]. The different

therapeutic responses of individual patients to specific APIs may be related to the presence of chronic diseases, individual genetic variations and environmental factors [100]. In order to develop personalized nanomedicine with maximal therapeutic efficacy and minimal side effects, it is necessary to consider the factors that often affect the biological fate of NPs in different patients. For example, some physical and mental states can increase the opsonization of NPs, and thus their reticuloendothelial system (RES)-uptake, causing fast blood clearance of NPs [101]. Other disorders, on the other hand, may disturb blood homeostasis and induce protein structure/amount variations leading to significant changes in the corona composition at the NP surface. In addition, chronic disorders can affect the intrinsic and natural elimination processes in the body. When cells interact with a foreign agent, or are subjected to specific conditions such as disease or stress, their secreted biomolecules can differ in a patient-specific-manner [102]. These secreted biomolecules have the ability to alter the protein corona composition, therefore one could reasonably expect a change in the biological fate of NPs when cells come into contact with foreign agents. In summary, human plasma from various individuals with different health conditions may have critical effects on the therapeutic and/or toxic impacts of corona-coated nanostructures (see Figure 3).

The development of effective nanotherapeutics will require that an understanding of the fundamental aspects of corona formation be coupled with studies that track the fate and biological effects/responses of NPs in different patients. To check the effects of various diseases on protein corona composition, Hajipour *et al.* [104] utilized two different types of commercial NPs (polystyrene and silica), which were incubated with plasma from human subjects with different medical conditions (e.g., breast cancer, diabetes, hypercholesterolemia, rheumatoid arthritis, fauvism, thalassemia, hemophilia A and B, the common cold, hypofibrinogenemia, smoking, hemodialysis and pregnancy). The results of this study suggest that corona composition at the surface of NPs depends quite strongly on the medical background of the patient. Supporting this idea, a different study found that graphene oxide sheets were shown to have different biological/toxicological responses (in terms of cellular toxicity, apoptosis and uptake, reactive oxygen species production, lipid peroxidation and nitrogen oxide levels) in the presence of coronas that were obtained from various types of diseases [103]. Based on these results, it can be concluded that a personalized protein corona

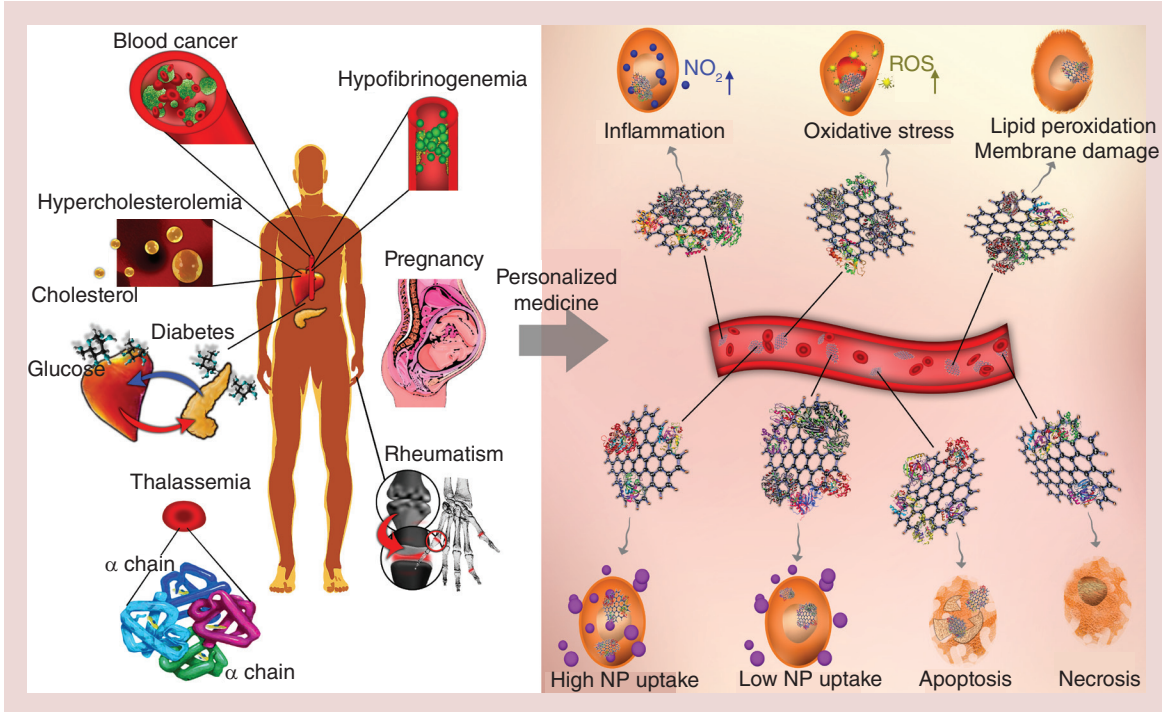

Figure 3. Schematic illustration of 'personalized medicine'. Various biological macromolecules in human patients with different disease/medical conditions can influence cell–nanoparticle interactions and the fates of the particles *in vivo*.
NP: Nanoparticle; ROS: Reactive oxygen species.
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will aid in the formation of effective and safe patient-specific NPs, tailored to the patient's disease-specific background, thus enhancing NP's clinical use.

Cell vision concept & its contributing factors

The cell vision concept states that various cells show different responses to the exact same types of NPs [33,105,106]. The different susceptibilities and/or resistance of cells to NPs is attributed to their distinct membrane properties, genetic background, defense/repair mechanisms, signaling pathways and growth rate. An example of this is the finding that mammalian cell lines (e.g. Vero, PK-15, and MRC-5) showed different degrees of susceptibility to gold NPs because of their diverse gene expression profiles [107]. The NP-cell interaction mechanisms and cell uptake level/route play crucial roles in determining the cytotoxic impacts of NPs. NPs interact differently with various cells and thus show different degrees of cell adherence/internalization efficacy as a function of the cell type [21]. Depending on the cell type, different antioxidant defense mechanisms are used to inhibit reactive oxygen species (ROS) production and maintain cell homeostasis. The most common strategies used to prevent ROS from exceeding the limit and/or to detoxify the excess ROS are antioxidant production, superoxide dismutase/catalase/peroxidase activation, and cell cycle retardation [108,109]. Chuang *et al.* [107] demonstrated that gold NPs killed AGS cells through induction of ROS production and consequent apoptosis, and only resulted in interruption of the cell cycle in A549 cells. The A549 cells minimized the harmful effects of ROS by activating several antioxidant defense mechanisms including ROS-exceeding prevention, excess ROS detoxification and cell cycle delay. The effects of inducing ROS production are also visible in cancer cells where, due to the already high oxidative stress level in cancer cells [110], even a slight increase in the ROS concentration may result in cell death/apoptosis. Ostrovsky *et al.* showed that ZnO NPs exert significant cytotoxic effects on breast and prostate cancer cells, but no toxic impacts on normal cells (breast and prostate cells) [111]. The cytotoxic effects of ZnO NPs are primarily related to their potency in inducing ROS through oxidative stress; these NPs can be used for the selective treatment of cancer cells. Graphene oxide selectively eradicated cancer stem cells, and showed no toxic effects against (nonstem) cancer cells [112]. This compound selectively kills cancer stem cells by inhibiting different STAT, WNT and NOTCH signaling pathways.

The physiological function of cells is another important parameter that regulates the susceptibility/resistance of cells to NPs. Sohaebuddin *et al.* showed that macrophages exhibited more susceptibility to multiwalled carbon nanotubes (MWCNTs), when compared with epithelial and fibroblast cells [113]. The authors speculated that the high susceptibility of the macrophages is attributed to their phagocytic function, which leads to the nonselective uptake of large amounts of NPs in a short amount of time. Because of this physiological function, and the early saturation of macrophages, the efficacy of NP internalization into epithelial and fibroblast cells was low, and the toxicological consequences of NPs were thus prevented. He *et al.* demonstrated that silica NPs (SNPs) exert no toxic or inflammatory effects on the organs (lungs, spleen, liver and kidneys) that are responsible for detoxifying/clearing the NPs [114]. Similarly, HepG2, which is derived from the liver, showed more resistance to SNPs than A549 and NIH/3T3 cells [115]. It has been previously shown that the cytotoxic impacts of SNPs are primarily dependent on the cell type [116]; the majority of NPs affect cell viability by disrupting membrane integrity. Therefore, because of their distinct membrane properties, the NIH/3T3 cells are more vulnerable to membrane disruption caused by SNPs than A549 and HepG2. Kim *et al.* [115] demonstrated that various cell lines, which are derived from the exact same cell type, behaved differently in the presence of identical NPs. In another study, the toxicity of starch-coated silver NPs were probed in normal human lung fibroblast cells (IMR–90) and human glioblastoma cells (U251). The particles caused DNA damage and cell cycle arrest in the G2/M phase (see Figure 4) [117]. More specifically, the induced G2/M phase arrest of the IMR–90–cells was much higher than that of U251 cells (compare Figure 5A & B), leading to the conclusion that induced cytotoxicity is strongly dependent on cell type. In addition, the IMR–90 cells demonstrated a concentration-dependent enhancement in DNA damage of up to 100 μg, whereas the U251 cells exhibited a steady increase of up to 400 μg (see Figure 4E for details). As a result of these findings, it should be concluded that the data obtained based on experiments from a specific cell type/line cannot be widely generalized.

Cell shape and mechanics are other determinants of NP uptake. Substrates with different mechanical and surface features have significant effects on the shape of the cultured cells [119–121]. For instance, $HepG_2$ cells formed different cell shapes when they were cultured on various substrates (i.e., polystyrene [PS], polystyrene sulfonate [PSS] and polyethylenimine [PEI]). Adherent cells on PEI showed a tendency to spread and cover a higher surface area than cells on PSS or PS. In this regard, round and spread-like forms of HepG2 cells had different uptake behaviors in response to 49 and 240-nm diameter polystyrene NPs [119]. In sum-

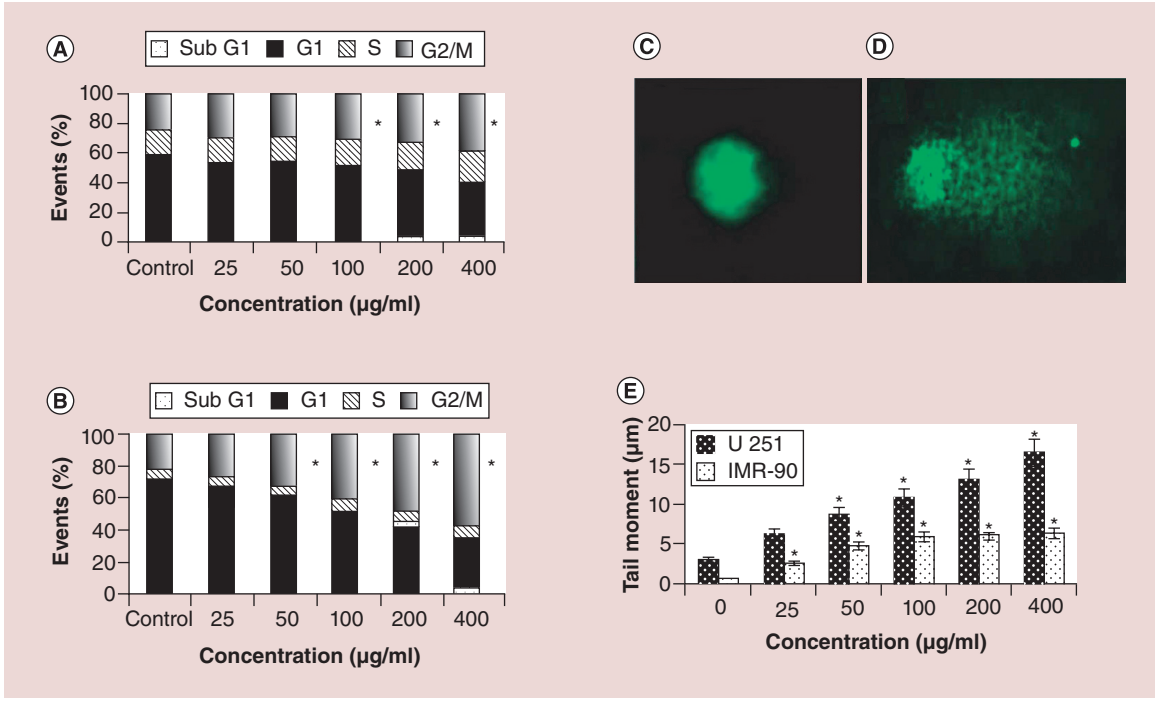

Figure 4. Toxicity evaluations of silver nanoparticles. Graphs showing the induction of G2/M arrest in **(A)** U251- and **(B)** IMR-90-cells after incubation with silver NPs at various concentrations. Comet analysis of the U251 cells **(C)** before and **(D)** after interactions with silver NPs. **(E)** The graph demonstrates the tail moments of DNA (µm). *Significant silver NP effects ($p < 0.05$).
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mary, to design safe NPs with high therapeutic efficacy, it is necessary to assess the biological impacts of NPs on the different cell types/ lines present within the target organ.

Cellular uptake & excretion of NPs

The cellular uptake of NPs can occur through a wide range of pathways such as phagocytosis, micro-pinocytosis, simple diffusion and receptor-dependent or independently mediated endocytosis [122,123]. One or a combination of uptake pathways can be operative, depending on the cell type, protein corona and physicochemical properties of the NPs [124]. Some cells may have particular endocytic routes that result in large differences in the amount and time of uptake. For example, adipocytes, fibroblasts, muscle and endothelial cells have a large number of caveolae and prefer this route for taking up NPs [125]. The uptake route also determines the intercellular accumulation and distribution of NPs and the consequent cellular response [126]. An example of this is the uptake of positively charged silica nanotubes (SNTs), relative to that of bare SNTs, which primarily occurs through fluid-phase endocytosis, leading to the accumulation of SNTs in lysosomal compartments [127]. Particle shape is also an important factor in determining uptake. Chithrani *et al.* showed that the uptake of spherical gold NPs is higher than that of their rod counterparts [17]. Furthermore, Warren *et al.* have reported the differential cellular uptake of gold nanoparticle aggregates of different sizes, versus single nanoparticles. In their work, the uptake patterns were significantly different for aggregated and non-aggregated NPs while also being cell dependent; the aggregated NP uptake was less than that of single NPs for HeLA and A549 cells and was higher for the MDA-MB 435 cell line [128].

NP excretion is a process of equal importance to uptake, and in turn depends on the properties of cells, particles, concentrations and time. The surface properties of NPs play an important role in their rate of excretion [129]. The exocytosis of NPs from the cells is likewise dependent on the cell type, NP concentration, incubation time and the cell medium components (BSA/serum/calcium); all factors that can affect the organelle distribution, intracellular localization/trafficking and the exocytosis pathways of NPs [130–135]. Recent reports have shown that when NPs are excreted from cells by exocytosis, their protein corona decoration is altered, and hence their *in vivo* behavior is modified – an important consideration for predicting long-term *in vivo* behavior [69]. The long-term retention of NPs inside the cells can lead to nanotoxicity, but can also increase the efficacy of diagnostic imaging, intracellular drug release and drug delivery systems [1,136–137]. For

example, the cytotoxic effects of mesoporous silica NPs were significantly increased through the inhibition of exocytosis by enhancing intracellular drug release [137].

In vivo challenges

In vivo studies of NPs provide new analytical challenges, as it is difficult to predict the effects of nanostructures on biological systems. After administration, NPs accumulate in certain areas of the body in a way that depends on several parameters including their size, shape, functionality, the animal model and administered doses, among others. The biodistribution and fate of NPs is usually modulated or altered by the protein cornea effect, the adsorption of biological

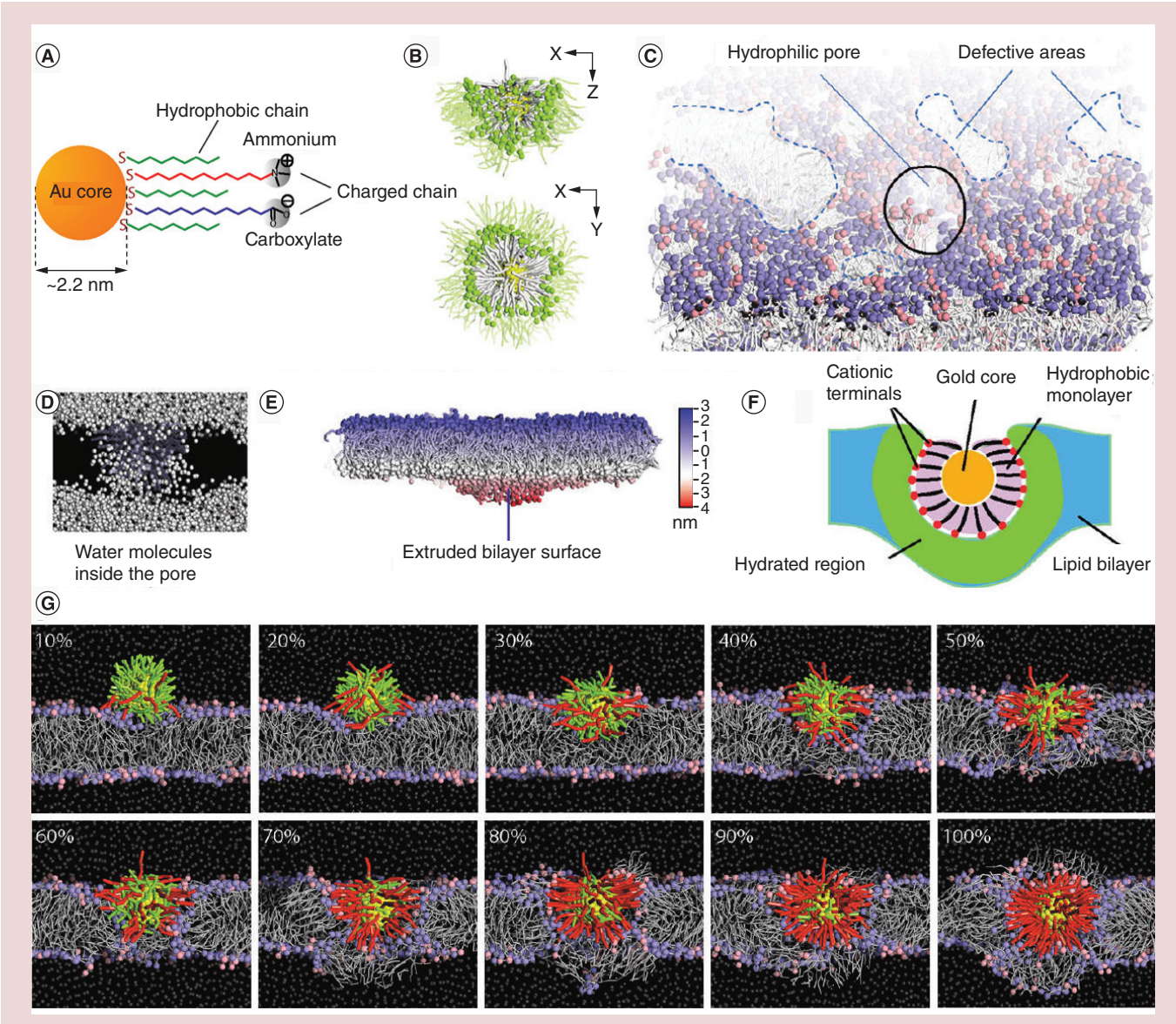

Figure 5. Structural changes in cellular membrane upon interaction with cationic gold nanoparticles. (A) A schematic representation of functionalized gold nanoparticles (NPs) with ammonium (positive) or carboxylate groups (negative). **(B)** The binding between the ligand terminals (at the surface of gold NPs) and the lipid head groups, which leads to the entrapment of gold NPs by the lipid shell. **(C)** The formation of porous and defective areas on the bilayer by penetration of the gold NPs. **(D)** The generated pores make the bilayer water permeable. **(E)** The lower leaflet of the lipid bilayer extrudes to facilitate the penetration of the gold NP. **(F)** A representative schematic showing the interface between gold NPs and the lipid bilayer. **(G)** Snapshots showing the interactions of gold NPs with different cationic surface charge densities (the percentage of cationic coverage is indicated in each snapshot) with a negative bilayer. The gold core is shown in yellow, hydrophobic ligands are in green, cationic ligands are in red, dipalmitoylphosphatidylcholine head groups are in ice blue, dipamitoylphosphatidylglycerol head groups are in pink, lipids tails are in silver, and water is in transparent white.
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molecules occurring on the NP surface. It is unclear whether the final biodistribution or localization of NPs is determined by the molecule that is adsorbed at the site of entry, by the one that is adsorbed during translocation in different parts of the body, or by a combination of both. Certain adsorbed proteins, such as opsonins (fibrinogen, IgG and complement factors), increase the possibility of phagocytosis and deletion of NPs by reticuloendothelial system cells, resulting in the accumulation of NPs in the spleen and liver [138–140]. However, when NPs bind to dysopsonin factors such as albumin, they have a longer circulation period in the blood [138,141,142]. Thus the original NPs may have a different *in vivo* metabolic trajectory because their surface characteristics could be completely altered due to the absorbed proteins. Furthermore, any labeling techniques, such as radiolabeling, may also change the surface properties of NPs and yield misleading results. The application of multi-indicator techniques in which the core NPs, absorbed protein and labeled molecules, have their own distinct markers would provide a complete picture of the metabolic processes pertaining to NPs [143].

Interference of NPs with conventional toxicity assays

Because of the poor correlation between data obtained from *in vitro* and *in vivo* cytotoxicity assessments, it is clear that there are several key factors to carefully consider in order to obtain reliable data [70,144]. Applying conventional cytotoxicity methods for the evaluation of NPs may result in significant unpredictable errors in toxicity results [145,146]. The reasons for the observed inaccuracies in the toxicological data may be related to the ability of NPs to change the cell medium components and thus interfere with the current *in vitro* toxicity assays [30]. For example, superparamagnetic iron oxide nanoparticles influence the cell medium by denaturing its proteins and adsorbing amino acids, vitamins and ions that can artifactually increase the observed toxicity [145]. We believe that the modification of conventional toxicity assays can be recognized as a promising strategy to obtain reliable toxicity results. An example of these modified assays is the use of a combined MTT/XTT (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) method that employs surface-saturated NPs [105]. In addition to the depletion effect described above, many NPs with unique electronic, optical, or magnetic properties (such as gold, silver, quantum dots, metal oxides and others) can interfere with conventional toxicity assays via complex and different mechanisms (see Table 1 for details) [29,30,64]. Kroll *et al.* and Marquis *et al.* reviewed NP interferences with assay components and the detection systems used in current nanotoxicology assays [29,30]. From the data presented in Table 1, and the predetermined reviews, one can see that the current toxicological assays, which have primarily been developed for drugs/biomolecules (and not for NPs), have certain limitations that have interfered with the assays' ability to produce completely accurate and reproducible nanotoxicology data [105]. To obtain precise nanotoxicological results there is a need to define new standard toxicological assays.

Advances in *in silico* nanotoxicology

In silico nanotoxicology is the integration of modern computing and information technology with molecular biology to predict the toxicity of nanomaterials [173,174]. In recent years, *in silico* studies have significantly progressed our understanding of NP toxicity. For example, molecular dynamics simulations were performed to probe the interactions of cationic and anionic gold NPs with electronegative and electroneutral bilayers [118]. The results showed that the positively charged NPs have a much stronger disruptive influence on the negative bilayers (see Figure 5), compared with their negatively charged counterparts. Furthermore, the levels of both membrane penetration and disruption depend on the surface charge density. At a cationic coverage of 50%, increasing the surface charges from a low degree up to the optimum resulted in enhanced membrane penetration. According to the findings from this simulation, one can expect that cationic NPs can induce significantly higher toxicities compared with anionic NPs. The primary reason for their higher toxicities is that the cationic NPs can pass the cell membrane and enter the cytoplasm through structural reconstruction, pore formation and phase transition [48,49]. These membrane perturbations can diminish the membrane's ability to control the nonspecific entrance of ions and other foreign extracellular bio-macromolecules into the cytosol, leading to extensive cell toxicity [21].

The future expansion of *in silico* nanotoxicology may lead to the development of high-throughput screening assays [175], as well as computerized statistical quantitative structure-activity relationship models (QSAR) that may help predict the *in vivo* fate of NPs [176]. The investigation of this field is already on the rise; for example, Sayes and Ivanov [177] have developed a mathematical model to correlate physical parameters such as size and zeta potential with biological activity (e.g., LDH release), in order to study cellular responses upon exposure to nanomaterials. Similarly, Puzyn *et al.* developed a nano-QSAR model to predict the cytotoxicity of 17 metal oxide NPs against *Escherichia coli* [178].

Table 1. Interference of nanoparticles with conventional toxicity assays.

Relevant toxicity end point	Process probed	Nanotoxicity assay	Interferences	False result	Remarks	Ref.
Necrosis	Membrane integrity	LDH	Inhibition of LDH	Carbon NPs increase false positive results	pH alteration affects LDH test	[147]
			Interferes with LDH enzyme	Copper ions and TiO_2 increase false positive results		[148,149]
		PI	Dye adsorption	Carbon NPs decrease markers of necrosis		[150]
				Gold NPs cause false positive results		
		Neutral Red	Dye adsorption	SWCNTs cause false positive results	pH alteration affects the intensity of light emissions	[147]
		Trypan Blue	–	–		
Apoptosis	Membrane structure	Annexin-V/PI	Adsorption of Ca^{2+}	Chitosan NPs adsorb Ca^{2+} and decrease annexin activity		[151]
			Adsorption of PI	Gold NPs cause fluorescent quenching		[150]
	Caspase activation	Gene expression and antibody-based assay	Inhibition of caspase 3	NPs containing trace metals and Zn^{2+} inhibit caspase-3 activity		[152]
	DNA breakage	TUNEL	–	–		[153]
Cell proliferation/ viability	Metabolic activity	MTT, WST–1, XTT	Depletion of free MTT and WST–1	SWCNTs cause false negative results	Adsorption of MTT is affected by pH alteration	[147]
			Interference with MTT reduction	Metal ions such as Zn^{2+} cause false negative results		[154]
			Interaction with tetrazolium salt	Ag NPs cause false positive results (more than 100% cell viability)		[155]
		Alamar Blue	Interaction with Alamar Blue	Nanoporous silicon may cause false results		[156]
			Interaction with dye	Carbon nanomaterials (SWCNT,C_{60}, carbon black, QD) cause false result		[157]
	DNA synthesis	Thymidine incorporation	–	–		[158]

NP: Nanoparticle; PI: Propidium iodide; QD: Quantum dot; SWCNT: Single-walled carbon nanotube.

Table 1. Interference of nanoparticles with conventional toxicity assays (cont.).

Relevant toxicity end point	Process probed	Nanotoxicity assay	Interferences	False result	Remarks	Ref.
Cell proliferation/ viability (cont.)	Colony formation	Colonogenic assay	–	–		[159]
Genotoxicity	DNA fragmentation	COMET Assay	Postfestum exposure interaction of DNA and particles	TiO_2 NPs cause false positive results		[160]
	Chromosome disorders	Chromosome aberration	–	–		[161]
	DNA mutation	8-hydroxy deoxyguanosine DNA adducts	–	–		[162]
Oxidative stress	ROS production	DCFDA hydrolysis	The cytochrome C released during apoptosis catalyzes the oxidation of DCFDA	NPs which induce apoptosis may cause false positive results	pH alteration affects the fluorescence	[163]
			Interference with optical density	Carbon-based NPs suppress DCF fluorescence and decrease markers of oxidative stress		[164]
		Rhodamine 123	–	–		[165]
	Lipid peroxidation	TBA assay for MDA	–	–		[162]
	SOD activity	Nitro blue tetrazolium	–	–		[166]
	SOD expression	Immunoblotting	–	–		[167]
	Linear strand Plasmid	Plasmid relaxation	DNA adsorption	NP may cause false negative results by adsorption of DNA		[168,169]
	The ratio of GSH to GSSG	HPLC (with DTNB)	–	–		[170]
Inflammatory responses	Determination of specific proteins	Immunoblotting	–	–	Concomitant contamination of NPs such as bacteria may induce inflammation	[158,171]
		ELISA	Adsorption of cytokines such as IL-8 and IL-6	Carbon and metal oxide NPs cause false negative results		

NP: Nanoparticle; PI: Propidium iodide; QD: Quantum dot; SWCNT: Single-walled carbon nanotube.

Table 1. Interference of nanoparticles with conventional toxicity assays (cont.).

Relevant toxicity end point	Process probed	Nanotoxicity assay	Interferences	False result	Remarks	Ref.
Inflammatory responses (cont.)		Western blot	–	–		
	Analyses of gene expression	Real-time PCR	–	–	The alteration of environmental conditions such as the pH and temperature, among others, and adsorption of RNA on the NP surface may lead to false results	[172]
		Microarrays	–	–		[33]

NP: Nanoparticle; PI: Propidium iodide; QD: Quantum dot; SWCNT: Single-walled carbon nanotube.

The ability of computational methods to add to *in vitro* studies is extremely beneficial, but it should not be forgotten that careful correlation of both *in vitro* and *in vivo* studies is required to provide effective predictions of *in vivo* behavior.

Conclusion

As it stands, there is conflicting data in the literature regarding the toxicity of NPs. In this review, we discussed the factors (e.g., protein coronas, cell types, local temperatures, NP dosage inside the cell, cell density and protein source) that cause this divergence. In addition, the intrinsic properties of NPs (biological reactivity, high adsorption affinity, catalytic activity and magnetic properties) were discussed, with an emphasis on their ability to interfere with conventional nanotoxicity assays. Based on the information presented in this paper, it should be concluded that interpretation of the current *in vitro* literature must be done with great attention to detail, especially regarding the use of proper experimental controls. In addition, investigators should keep in mind that different cells handle the toxicity of NPs differently. To this end, exact information on NP characterization, including the aforementioned physical and functional factors, should be provided by nanotoxicology-based reports. As such, one must know the cytotoxicity results for each cell type and refrain from generalizing to others. Researchers should also be very careful when conducting nanotoxicity assay selection; a suitable assay should not have any interactions with the NPs. For instance, an example of a reasonable control for an assay is a lack of response of the assay to NPs, in the absence of cells.

Future perspective

Considering all of the aforementioned factors should lead to more consistency between the results from different laboratories. Furthermore, attention paid to these key issues provides the potential for developing *in vitro* assays that can more accurately predict *in vivo* behavior – an outcome that would greatly speed up the development of new nanotherapeutic agents. Moving forward, researchers in the field of nanotoxicology should aim to conduct more research *in vivo,* in order to conduct better toxicological and therapeutic dose assessments. To that end, there are numerous analyses, which should be standardized and comprehensively considered in animal studies including animal behavioral evaluation, careful monitoring of the complete blood count, serum chemistry and urinalysis. In addition, developmental, reproductive and carcinogenic toxicities should be clearly assessed. Choice of organs sampled should also be standard-

ized depending on both the type of NPs and route of administration.

Besides animal studies, we suggest the use of *in silico* nanotoxicology (described above) and 'organ-on-a-chip' microdevices (a very recent major development in nanotoxicology which aims to mimic the complex physiology of various organ systems using cell-based biochips) [179] to enhance the capabilities of cell culture models to provide accurate, high-throughput and cost–effective semi-*in-vivo* stages for toxicology applications. Besides the significant scientific outcome of 'organ-on-a-chip' microdevices, the progress in the field may someday eliminate the need for animal testing, which would greatly enhance the capability of researchers to conduct meaningful studies.

It is clear that nanoscience, and the use of nanotechnologies in medicine, is rapidly becoming a huge target for medical diagnostics and therapeutics. In light of these advancements, researchers should pay particular attention to the challenges faced when conducting reliable nanotoxicology assays. While more reliable means of *in vivo* or semi-*in vivo* are currently being investigated, the attention to detail within *in vitro* studies should not be forgotten. Researchers should aim to identify all aspects contributing to the potential toxicity of an NP (whether it is physical characteristics, intracellular or intercellular interactions) and investigate them thoroughly in order to gain a more holistic view of the toxic effects of a NP.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Moving towards reliable assessments of nanomaterial toxicity

- The physicochemical properties of nanomaterials (i.e., the size, shape, charge, stability, solubility, chemical composition, surface modification, agglomeration and sedimentation states) should be fully characterized.
- The supernatant of the nanomaterials should be analyzed as a control sample.
- The release of toxic ions/molecules from the surfaces of nanomaterials should be carefully monitored and reported.

Cell culture assays & nanotoxicology

- Cell vision effects should be considered in the interpretation of toxicity results.
- One should clearly report the cell culture type (e.g., 2D and 3D; cell numbers).
- The excretion of nanomaterials from cells should be tightly measured and reported.

Insights into protein coronas & nanotoxicology

- Temperature (gradient) changes have a significant role in the composition of protein coronas and cell responses. Thus, a tight control on temperature should be applied during experimentation.
- Protein sources (disease-specific) can change the decoration of the protein corona. Therefore, complete and accurate information about the plasma/serum (e.g., diseases type, among others) should be provided in toxicological reports.

Key factor in conventional toxicological assays

- The reported pitfalls in the conventional approaches must be carefully reviewed to select suitable methods for specific types of nanomaterials.

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