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The Challenge To Relate the Physicochemical Properties of Colloidal Nanoparticles to Their Cytotoxicity

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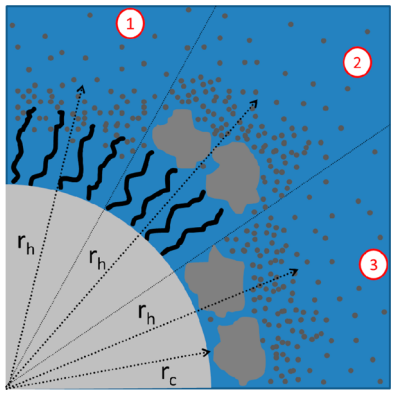
CONSPECTUS

Nanomaterials offer opportunities to construct novel compounds for many different fields. Applications include devices for energy, including solar cells, batteries, and fuel cells, and for health, including contrast agents and mediators for photodynamic therapy and hyperthermia. Despite these promising applications, any new class of materials also bears a potential risk for human health and the environment. The advantages and innovations of these materials must be thoroughly compared against risks to evaluate each new nanomaterial. Although nanomaterials are often used intentionally, they can also be released unintentionally either inside the human body, through wearing of a prosthesis or the inhalation of fumes, or into the environment, through mechanical wear or chemical powder waste. This possibility adds to the importance of understanding potential risks from these materials.



Because of fundamental differences in nanomaterials, sound risk assessment currently requires that researchers perform toxicology studies on each new nanomaterial. However, if toxicity could be correlated to the basic physicochemical properties of nanomaterials, those relationships could allow researchers to predict potential risks and design nanomaterials with minimum toxicity.

In this Account we describe the physicochemical properties of nanoparticles (NPs) and how they can be determined and discuss their general importance for cytotoxicity. For simplicity, we focus primarily on in vitro toxicology that examines the interaction of living cells with engineered colloidal NPs with an inorganic core. Serious risk assessment of NPs will require additional in vivo studies. Basic physicochemical properties of nanoparticulate materials include colloidal stability, purity, inertness, size, shape, charge, and their ability to adsorb environmental compounds such as proteins. Unfortunately, the correlation of these properties with toxicity is not straightforward. First, for NPs released either unintentionally or intentionally, it can be difficult to pinpoint these properties in the materials. Therefore, researchers typically use NP models with better defined properties, which don't include the full complexity of most industrially relevant materials. In addition, many of these properties are strongly mutually connected. Therefore, it can be difficult to vary individual properties in NP models while keeping the others constant.

1. Introduction

The definition of nanoparticulate materials is relatively broad, and thus, to simplify, this Account focuses on engineered colloidal nanoparticles (NPs) with an inorganic core. However, in particular in biological environments (e.g., waterbodies, cell culture media, body fluids such as blood, plasma, interstitial fluid, urine, *etc.*), these NPs are hybrid materials with an inorganic core and an organic

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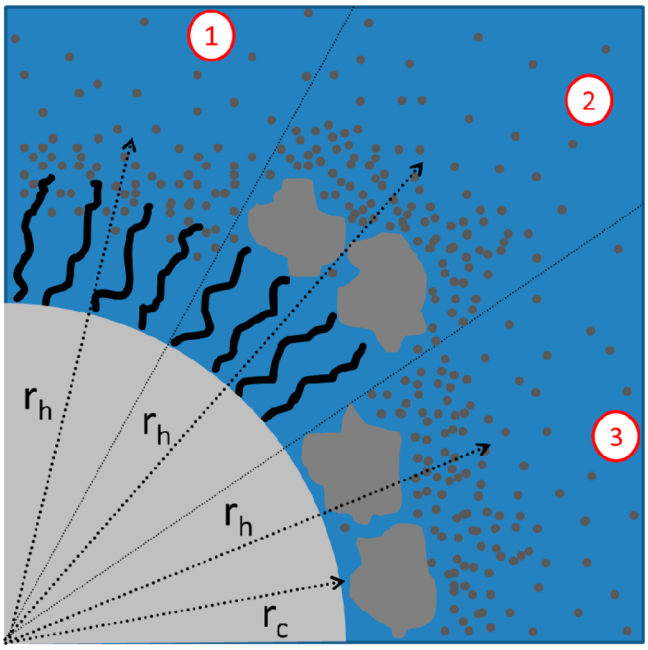

FIGURE 1. Geometry of a NP as characterized by its core diameter r_c and hydrodynamic diameter r_h. The inorganic core (drawn in light gray) can be coated by organic ligands (drawn in black, scenarios (1) and (2)). At any rate, counterions from solution (drawn as small points in dark gray) are attracted by the NP. In biological media, molecules such as proteins adsorb to the NP surface (drawn in dark gray, scenarios (2) and (3)).

coating. In biological media, constituents of the media will generally adsorb to the NP surface, thus leading to NPs with an organic shell (cf. section 6).[1] Also for *in vivo* scenarios, such as abrasion of nanoparticulate material from prosthesis,[2] the original NPs (i.e., the wear debris) may be of purely inorganic nature, but in contact with body fluids, they will be covered with organic molecules, in particular proteins, the so-called protein corona (cf. section 6).[3] At any rate, any NP will comprise an (in this review) inorganic core and an (in general complex) organic shell. A sketch of the NP geometry is given in Figure 1. The hybrid nature of NPs involves several fundamental challenges in defining basic properties of NPs, such as dose (cf. section 2) and size (cf. section 4), which will be discussed later. In addition, cytotoxic effects of the NPs can potentially arise from the core and/or from the surface coating. Though the surface coating interfaces the NP with its biological environment and thus controls, for example, the pathway of cellular internalization of the NP, toxicity can arise from the core and/or the shell. Both may change over time, the core mainly by corrosion (i.e., leaching of ions, cf. section 3) and the organic shell by biodegradation (in particular after cellular uptake) and/or rearrangement of the protein corona (cf. section 6).[4] In particular, *in vitro* or *in vivo* internalization may significantly alter NP properties and thus influence cytotoxic effects, which so far has been only poorly investigated. For this reason it does not make any sense to claim that NPs of a certain inorganic material are toxic or nontoxic, as toxicity will also depend on the surface coating and not only on the material of the inorganic core. A simple example is CdSe NPs (so-called quantum dots), where toxicity for NPs with identical inorganic CdSe cores is strongly influenced by the surface coating.[5] Also shape-controlled elongated Au NPs capped by cetyl trimethylammonium bromide (CTAB) are toxic due to the shell of CTAB and not due to the inorganic Au core.[6] In turn, it would be wrong to say that toxicity is determined by the surface coating, which ultimately mediates interaction with cells. Experiments with NPs with different cores (Au, FePt, no core) but the same surface coating (a carboxy-terminated polymer) gave an example in which the core (Au, FePt), but not the surface coating (i.e., polymer micelles without inorganic core inside), was responsible for cytotoxic effects.[7] Thus, for any cytotoxicity study the hybrid nature of NPs must be taken into account, and by suitable control experiments the origin of toxic effects needs to be determined, which can be the inorganic core and/or the organic surface.

2. Dose and *in Vitro*/*in Vivo* Models

Already in the medieval ages, Paracelsus pointed out that toxic effects depend on the administered dose. As nowadays many researchers are looking for cytotoxic effects, inglorious examples can be found in the literature in which the mass of NP material injected in an animal virtually has the same mass as the blood of the animal itself, whose consequent death certainly does not qualify for claiming these NPs to be cytotoxic. This example visualizes the importance to quantify the administered dose of NPs, which due to their hybrid properties (cf. section 1) is no trivial task. The easiest way of quantifying NP doses is *via* mass concentrations, i.e. the mass of NP material per solution volume (in mg/L). Assuming the NP solution is purified and does not contain anything but the NPs (cf. section 3), the mass of the NPs dispersed in a solution of certain volume can be determined by evaporating the solvent and weighting the NPs. The intrinsic problem of mass concentrations comes into play when toxicities of NPs with different surface coatings are to be compared. This could involve a comparison of NPs with identical inorganic cores but with different surface coatings. One surface coating could be a monolayer of surfactants, while the other surface coating would involve, in addition to the monolayer, large attached polymers. Due to the NPs'

hybrid nature, the mass of the second type of NPs thus is bigger than the one of the first type of NPs. This effect can be quite dramatic due to the high surface-to volume ratio of small NPs. For instance, one naked Au core with a diameter of 4 nm has a mass of ca. 65×10^{-23} kg, whereas the same core conjugated with 15 molecules of polyethylene glycol (PEG) with molecular weight of 20 kDa (mass of the PEG shell of ca. 50×10^{-23} kg) would have a mass of ca. 115×10^{-23} kg, which is a significant change. In other words, in case comparison of toxic effects is performed with solutions with the same mass concentration, solutions of the NPs with polymer would comprise fewer NPs in number than solutions with NPs without the polymer. Alternatively, the dose can be given in terms of concentrations, i.e. in moles of NPs or number of NPs per volume of solution (in mol/L or L^{-1}). Mass concentration (mg/L) and concentration (mol/L) are correlated by the molecular weight of one NP as a proportionality factor. Thus, the mass of NPs could be converted into the number of NPs, in case the molecular weights of the NPs were known. Unfortunately, the hybrid nature of NPs (cf. section 1) makes a determination of the molecular weight (which would involve the contribution of core and shell) quite cumbersome. Instead of quantifying NPs by their concentration, also the number of inorganic atoms (from the core) per volume of solution (in mol/L or L^{-1}) can be specified. The number of inorganic atoms per NP core (and thus the correlation factor between both entities) can be experimentally estimated by deriving the volume V_c of the NP core from the NP's dimensions (e.g., for a spherical geometry, the core diameter d_c) as determined by imaging techniques such as transmission electron microscopy (TEM). Knowing V_c and the density ρ of the NP core material, the mass of one NP core can be calculated as $m_c = \rho V_c$. Considering an inorganic core of the composition A_xB_y (e.g., Fe_2O_3), the mass of one molecule m_m of A_xB_y can be calculated as x-times the atomic mass of element A and y-times the atomic mass of element B (e.g., $m_m(Fe_2O_3) = 2m_m(Fe) + 3m_m(O) = 2.6 \times 10^{-25}$ kg). The number of atoms A or B per NP of A_xB_y thus is x-times or y-times the coefficient m_c/m_m (e.g., each Fe_2O_3 NP with $d_c = 11$ nm core diameter, $\rho(Fe_2O_3) = 5.25$ g/cm^3, comprises ca. 28000 atoms of Fe). The atomic concentration of one element in solution (mol/L) can be determined for example by inductively coupled-plasma mass-spectrometry (ICP-MS),[8] and thus by knowing the number of atoms per NP, the NP concentration can be derived. Alternatively, for corrosive NPs (such as CdSe and Ag, cf. section 3) which release toxic ions from their inorganic core, the number of surface atoms (i.e., the atoms on the surface of the inorganic core which are most likely released upon corrosion) can also be specified.[5] In some special cases (such as quantum dots and plasmonic NPs), NP concentrations (mol/L) can be determined from absorption measurements.[9,10] Knowing about the different approaches to specify doses, to allow comparison among studies, the way NP concentrations have been determined should be precisely described. Logically also, the *in vitro*/*in vivo* model used highly influences experimental results. *In vitro* and *in vivo* nanotoxicity is generally triggered by the induction of oxidative stress by free radical formation, i.e. the creation of radical oxygen species (ROS), following the administration of NPs. A detailed list of cytotoxic effects of NPs (involving besides ROS also others such as membrane disruption) can be found in ref 11. In the case of *in vivo* sequestration of NPs by phagocytic cells in the organs of the mononuclear phagocyte system, organs such as liver and spleen are the main targets of oxidative stress. Other organs exposed to high blood flow, such as the kidneys and lungs, are also subject to oxidative stress. Toxicity certainly is correlated with NP uptake. A great risk is that NPs can accumulate *in vivo* and stay in animals over extended periods of time. Also *in vitro*, there are first studies which demonstrate that NPs which have been incorporated by cells are more harmful than those remaining in the surrounding medium.[12] In fact, different uptake routes of different cell types affect the toxicity of NPs.[13] Internalized CdTe NPs have been for example demonstrated to be more toxic than the corresponding amount of free Cd^{2+} ions in an in vivo model.[14] Besides the type of cells,[13] toxic effects depend also on the state of cellular differentiation.[10] Coming back to the problem of defining administered NP doses, the geometry of NP exposure also plays a role *in vitro*. In particular, when NPs are not homogeneously dispersed in the medium but sediment on the cells due to low colloidal stability (cf. section 3), a thin film of medium over a big area of cells will have a different effect than a high film of medium over a small area of cells.[15] Thus also, the interplay between NP concentration and the cellular density depends on the exposure geometry and has to be considered.

3. Colloidal Stability, Purity, and Inertness

Arguably, colloidal stability is the key physicochemical parameter of NP dispersions, as it influences most of the other parameters, and thus has a great effect on cytotoxic effects.[16] NPs tend to agglomerate due to van der Waals attraction. To introduce repulsion, NP surfaces typically are modified with charge or with polymer brushes. These organic coatings

provide colloidal stability by electrostatic (equally charged NPs repel each other) or steric (polymer brushes cannot completely penetrate each other) repulsion. The inorganic cores of NPs can be synthesized from a large variety of materials, and many different surface coatings for provision of colloidal stability have been reported.[10] The quality of the surface coating for providing colloidal stability can be relatively easy probed by measurements of the hydrodynamic diameter of the NPs (cf. section 4).[17] Dramatic deviations of the hydrodynamic diameter (d_h) relative to d_c can be ascribed typically to agglomeration. Importantly, colloidal stability probed only in water is not a sufficient indicator. Salts screen charges on the NP surface and thus reduce electrostatic repulsion, as well as proteins from biological environments will adsorb on the NP surface (cf. section 6).[18,19] Thus, measurements of d_h should be performed in the media which the NPs experience after administration.[20] Again, the dynamic nature of the NP hybrid geometry (cf. section 1) needs to be pointed out, which involves that colloidal stability of NPs may change during their life cycle.[21] It is a rule of thumb that NPs which possess poor colloidal stability and which are agglomerated will remain irreversibly like that. Obviously agglomeration increases the effective size of NPs (cf. section 4). Agglomeration due to limited colloidal stability can thus smear out effects of different NP sizes.[22] Also ζ-potential measurements, which are typically used to quantify the surface charge of NPs (cf. section 5) are strongly affected by agglomeration.[17] Colloidal stability is also paramount for purification of NP solutions from residual reactants or leached constituents. Cytotoxicity studies should be performed with pure NP solutions to make sure that effects are related with the NPs and not with impurities. Ultrafiltration, size exclusion chromatography, (gel) electrophoresis, and flow field fractionation are established methods for NP purification.[23,24] However, in the case of limited colloidal stability, the NPs may irreversibly precipitate upon purification, for example due to partly stripping off the organic surface layer or by screening of surface charge in the required buffer solutions. Considering pure NP solutions, the inorganic NP cores can trigger cytotoxic effects in two ways, either due to reaction of their surface with the environment or by leaching toxic ions, which in both cases leads to the production of ROS.[13] Corrosion of NPs leading to the release of toxic ions to solution has been reported for several NP materials, such as Ag,[25] CdSe,[5] Cu and CuO, NiO,[26] and ZnO.[27] In these cases, cytotoxicity (e.g., the ability to generate ROS) has been correlated with the corrosiveness (i.e., the rate of dissolution) of the NPs. Thus, controls are required to determine whether toxicity is due to released ions or the NPs by themselves.

4. Influence of Size and Shape

Due to their hybrid character, the size of NPs is not a trivial issue (cf. section 1). The NP core size (e.g., d_c in a spherical geometry) can be determined by TEM. This is due to the fact that the organic shell around the core provides at best low contrast in TEM images, and thus, the core can be unequivocally identified. In solution the hydrodynamic diameter d_h can also be determined. This is technically derived from measurements of the diffusion constant D and the Stokes–Einstein equation $D = (k_B T)/(3\pi\eta d_h)$, where k_B and η are the Boltzmann constant and the viscosity of the solvent, respectively. Typical techniques for determining d_h in this way are dynamic light scattering (DLS)[17] or fluorescence correlation spectroscopy (FCS). Though DLS is among the most used techniques for particle sizing, it is generally considered a ``low resolution'' method[17] and is limited to thorough filtering to avoid spurious signals, the possible interference when the sample absorbs the laser light, or the practical impossibility to reliably measure sizes below 10 nm. The hydrodynamic radius comprises the organic surfactant layer (cf. Figure 1), intentionally attached molecules (bioconjugation; cf. section 1), the adsorbed protein corona (cf. section 6), and the cloud of attracted counterions, and thus, it is always is bigger than d_c. In comparative studies in which size is kept constant and other parameters are varied, it is important to discuss which diameter (d_h or d_c) is kept constant. Typically, the cytotoxicity of NPs is size-dependent, and in general, the smaller the NP, the higher its cytotoxicity.[28] This effect can originate from an increasing reactive surface area of smaller NPs, though in some cases also from a size-dependent uptake rate. As the size of a NP decreases, its surface area-to-volume ratio increases and also allows a greater proportion of its atoms or molecules to be exposed to the exterior. Shrinkage in size may create discontinuous crystal planes that increase the number of structural defects, as well as it may disrupt the well structured electronic configuration of the material, so as to give rise to altered electronic properties. This could, for example, establish specific surface groups that could function as reactive sites. Therefore, it is more appropriate in some cases to quantify NP dose in terms of reactive surface area (i.e., number of surface atoms) rather than in number of NPs (i.e., total number of atoms; cf. section 2).[28] In case cytotoxicity takes place in the intracellular space, it will be dependent on the NP uptake rate.[29] In general, bigger NPs

are less efficiently and more slowly incorporated by cells. To investigate this, not only EC_{50}-values (i.e., the mean effective concentration at which half of the cells are impaired) but also the kinetics of uptake (i.e., how fast are the NPs incorporated) should be determined. Extensive *in vitro* studies of size-dependent uptake have been performed for Au NPs[30] and SiO_2 NPs.[31] Also, the mechanism for cytotoxic effects can change with NP size.[32] *In vivo* NP size influences the biodistribution of administered NPs.[33] Many organs of the human body act as size-dependent filters. Large NPs (>200 nm) will be easily detected by the immune system, removed from the blood, and delivered to the liver and the spleen.[34] Very small NPs (<5−15 nm) can be excreted through the kidneys by renal filtration.[35] Therefore, for example, the optimal NP size for intravenous (i.v.) delivery ranges between 10 and 100 nm, as these NPs will have (provided an appropriate surface chemistry) the longest blood circulation time. However, the values for threshold NP sizes have to be interpreted with care, and they are valid only for nonagglomerated NPs. NP shape can also influence NP uptake[36] and toxicity.[37]

5. Role of Charge

Charge can warrant for colloidal stability (cf. section 3) and thus prevent NP agglomeration. Charge also regulates interaction of NPs with the biological environment, such as adsorption of serum proteins (which may trigger uptake by endocytic pathways,[13] or it even may change the protein conformation[38] (cf. section 6)) and NP incorporation by cells (i.e., uptake rate and pathway of internalization).[39] Practically, charge is often quantified in terms of ζ-potential [mV]. The Poisson equation for electric fields relates charge [C] and electric potential [mV]. However, ζ- potentials cannot be trivially converted into surface charge densities. This is first due to the complex hybrid geometry of NPs (cf. section 1) and second due to what can be experimentally detected (ζ-potential). NPs in solution can absorb ions (Helmholtz layer), and charged objects in electrolytic solution are in addition screened by counter charges (i.e., diffusive ions).[40] Whereas the surface potential is the electric potential at the NP surface (and thus directly corresponds to the surface charge), the ζ-potential is the electric potential in the distance of the screening length (i.e., the decay length of the diffusive cloud of counterions) from the NP surface. As ions can absorb to the NP surface, the NP surface charge depends on the medium in which the NPs are dispersed (e.g., the surface charge of carboxyl terminated NPs will depend on pH due to the equilibrium $-COOH \leftrightarrow -COO^- + H^+$). As in addition the screening length of the diffusive ion cloud depends on the ion concentration and the ion valency, the ζ-potential is strongly influenced by the surrounding medium. In the context of *in vitro* studies, cationic NPs are believed to be more toxic to cells than their neutral or anionic counterparts.[11] As the cellular lipid bilayer carries a net negative charge, it is easier for cationic NPs to attach to the cellular surface and thus to be internalized.[41] It is quite possible, for this reason, that higher cytotoxicity of positively charged (cationic) NPs is often a simple effect of higher uptake rate. However, details are more complex.[42] Distinct mechanisms have been proposed to explain the cytotoxicity of cationic NPs, such as strong interaction of cationic NPs with the cell membrane, leading to hole formation, membrane thinning and/or erosion,[42] damage to the acidifying endosomal compartments by the proton sponge effect, followed by mitochondrial injury,[13] increase of intracellular Ca^{2+} concentration following membrane depolarization,[41] or release of cytotoxic surfactants such as the widely used CTAB (cf. section 3).[6] However, as pointed out repeatedly, most physical−chemical parameters are entangled. Effects of charge may be correlated with size-dependent uptake, and additional surface coatings such as PEG introduce complexity. PEGylated neutral NPs, for example, show nearly no interaction with some cells.[43] Also, *in vivo* studies show that PEGylated NPs have a longer retention time in blood than their charged counterparts and the biodistribution of charged NPs in organs, tissues, and excretion is also charge dependent.[44] This is thought to be connected with reduced interaction of PEGylated NPs with serum proteins, which makes them "invisible" to the phagocytic system.[43]

6. Adsorption of Environmental Molecules (in Particular Proteins) to the NP Surface and Sterilization

NPs in physiological media will absorb proteins, forming the so known protein corona, in particular, if they do not possess passivating surface coatings such as PEG.[19] By this process the mononuclear phagocyte system can recognize easily intruded NPs in the body. In general, if a NP is not well-passivated by a surface that prevents nonspecific protein adsorption, opsonization occurs, and most probably the complement system will be activated, producing an immunological response against them.[45] Typically, this immunological response will lead to accumulation of NPs in the liver and spleen. The surface charge and size of NPs influences interaction with proteins of the physiological medium.[18,46] Adsorption of proteins can directly trigger cytotoxic effects, or indirectly by enhancing NP uptake.[30,38] Concerning NP

uptake, the adsorption of serum proteins can hide the synthetic organic layer of the NP surface and lead to nonspecific uptake of the NPs into cells by receptor mediated endocytosis. Protein adsorption onto nanoparticles can also adversely affect the function and structure of proteins.[38,47] Also, special attention should be paid to sterilization techniques of colloidal solutions containing NPs, as these can prevent the presence (absorbed or coexisting) of microbial life in solutions of NPs. Before challenging NPs with any living organism, these should be sterile. Otherwise, toxicity results might be misleading (i.e., toxicity and/or infection resulting from coexisting microbial life).[48]

7. Conclusions

Nowadays it is still not possible to reliably break down the cytotoxicity of NPs to their physicochemical properties, as they are strongly interconnected. Model NPs in which exclusively only one parameter can be varied are very hard to synthesize on a general basis. As different cell types have different function, also their interaction with NPs can differ significantly. As a general rule of thumb, small positively charged NPs are more toxic than big negatively charged ones, though this may not be true under particular conditions. The most promising directions in the future will include high throughput screening assays which allow for large scale comparison of huge sets of well-defined model NPs in which properties are systematically varied.

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FOOTNOTES

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