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Toxicity of Engineered Nanomaterials: A Physicochemical Perspective

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Abstract

The global market for nanomaterial-based products was forecasted to reach 100 billion dollars per annum for 2011–2015. Extensive manufacturing and the use of engineered nanomaterials (ENM) have raised concerns regarding their impact on biological response in living organisms, and the environment at large. The fundamental properties of nanomaterials exhibit a complex dependence upon several factors such as their morphology, size, defects, chemical stability, etc. Therefore, it is exceedingly difficult to correlate their biological response with their intricate physicochemical properties. For example, varying toxic response may ensue due to different methods of nanomaterial preparation, dissimilar impurities and defects. In this review, we surveyed the existing literature on the dependence of cytotoxicity on physicochemical properties. We found that ENM size, shape, defect density, physicochemical stability and surface modification to be the main causes that elicit altered physiological response or cytotoxicity.

Keywords

nanoparticle; protein corona; nanotube; graphene; gold; silver; zinc

Introduction

In the last two decades, the ability to tailor and engineer nanomaterials with unique physicochemical properties has opened new avenues for numerous applications in electronic, chemical, biological, and medical fields. The small size of engineered nanomaterials (ENM) leads to high surface area and thus results in altered chemical reactivity compared to its bulk counterparts. For this reason, several consumer products such as paints, medicines, cosmetics etc. are beginning to incorporate ENM to increase the overall product quality. In fact, the global market for ENM based products was forecasted to reach 100 billion dollars per annum for 2011–2015 [1]. Extensive manufacturing and the use of ENM have raised concerns regarding their impact on biological responses in living organisms, and the environment at large [2–26]. The fundamental properties of ENM exhibit a complex dependence upon several factors such as their morphology, size, defects, chemical stability, etc. Therefore, it is exceedingly difficult to correlate ENM's biological response with its intricate physicochemical properties. For example, varying toxic response may ensue due to different methods of ENM preparation, dissimilar impurities and defects [27]. Hence, an appropriate analysis of the observed biological response or cytotoxicity demands a need for excellent *in vivo* and *in vitro* characterization of nanomaterial properties.

It has been widely accepted that much more research is needed for: i) identifying the list of physicochemical properties for evaluation of cytotoxicity and ii) correlating the listed physical characteristics to the biological response. In the past decade, ENM induced cytotoxicity has been the central theme of several reviews, debates and research reports [2–26]. However, the existing research has revealed many inconsistencies and the results have been largely inconclusive. For example, carbon nanotubes were found to be a potential health hazard in some studies while others have reported low or no toxicity [27–30]. There is compelling evidence to suggest that CNT length, diameter and defect density affects cytotoxicity [27, 31, 32]. Further, many of ENM are inherently hydrophobic in nature and are often coated with biologically compatible materials (such as the proteins available in cell culture media for *in vitro* studies and plasma proteins in *in vivo* studies) forming a “corona” [33–37]. This corona itself is known to screen the surface properties of ENM and thus result in a varying physiological response. Thus, it is crucial to appropriately characterize both the as prepared and media coated ENM to effectively correlate the physiological response with physicochemical properties.

In this article, we review the dependence of cytotoxicity on ENM physicochemical properties. We discuss the results from previous studies in which carbon based nanomaterials (spherical fullerenes, sheets of graphene and single- & multi-walled nanotubes) and other metal & metal oxide nanoparticles (Au, Ag, TiO₂ and ZnO) were used. Specifically, the dependence of toxicity, inflammatory response, bio-distribution upon: i) ENM size and shape, ii) physicochemical stability, iii) defects and impurities and iv) surface modification.

Dependence of biological response on physicochemical properties

Effect of Size and Shape

The electronic and optical properties of ENM can be tuned for any desired application by controlling their size and shape [38]. For example, different Au nanostructures (with different shapes and sizes) exhibit surface plasmon resonance at different wavelengths. The control of ENM size and shape is at the heart of nanotechnology applications. Importantly, the size of nanoparticle dictates its interactions with biological systems, including absorption, diffusion and biodistribution. Thus, it is pertinent to understand the dependence of biological response on the size and shape of ENM. Chithrani *et al.* observed that the cellular uptake of Au nanoparticles (NPs) was greatly dependent upon size [39]. In their studies, the maximum uptake of Au NPs by HeLA cells was found to occur at a size of 50 nm. Likewise, Jiang *et al.* showed that different size Au and Ag nanoparticles coated with antibodies exhibit the ability to regulate the process of membrane receptor internalization [40]. Importantly, 40- and 50- nm Au nanoparticles exhibited the greatest toxicity in their studies concurring with Chithrani *et al.* Similarly, Osaki *et al.* observed that 50 nm CdSe nanoparticles entered cells via receptor-mediated endocytosis more efficiently than smaller nanoparticles [41]. Such similar results, obtained in contrasting systems (CdSe and Au NPs), suggest that the maximum NP uptake occurs at an optimal size around 50 nm. In fact, the size of some viruses and lipid-carrying proteins that undergo efficient endocytosis falls within the same range. Interestingly, Wang *et al.* found that the Au NPs did not exhibit any size-dependent toxicity as suggested by Chithrani and Osaki *et al.* They attributed the absence of any toxicity to the possible aggregation of Au NPs outside the cell [42]. This aggregation primarily resulted from the presence of Na salts in the DMEM media. In addition to size-dependence, Chithrani *et al.* observed that the uptake of Au nanorods is lower than Au NPs. The cellular uptake of rod-shaped structures with lower aspect ratio (1:3) is greater than higher aspect ratio (1:5) nanoparticles [39].

As in the case of Au, Ag and CdSe nanoparticles, the aspect ratio of CNTs has been found to influence their interaction with macrophages and the ensuing immunological response. For instance, low aspect ratio MWNTs (~220 nm in length and 25 nm in diameter) were enveloped by macrophages and phagocytes more readily relative to the longer MWNTs (~825 nm in length and 25 nm in diameter) [43]. Sato *et al.* found that low aspect ratio MWNTs (~220 nm in length and 25 nm in diameter) injected into subcutaneous tissue in rats were in the cytosol of macrophages for > 4 weeks, while the high aspect ratio MWNTs (~825 nm in length and 25 nm in diameter) were not found in macrophages and resulted in more inflammation [43]. Thus, longer MWNTs cause more toxicity and cytokine production than the shorter MWNTs.

Poland *et al.* demonstrated that MWNTs exhibit length-dependent asbestos-like pathogenic behavior [44]. However, the nontoxic length scales asserted by Poland *et al.* is significantly different from Sato *et al.* This difference may be attributed to the defects along the MWNT length and their influence on toxicity [27]. Firme *et al.* suggested that it would then be helpful to isolate MWNTs with similar charge, defect densities and lengths by using an electrophoresis-based process prior to any testing. Such precautions steps are proposed to reduce the apparent inconsistencies in the reported literature.

Effect of physicochemical stability

Many of the ENMs are inherently hydrophobic and as a result they do not form stable suspensions in aqueous media. For this reason, they are often stabilized using surfactants, functional groups or polymer coating [27]. For example, Au NPs are often functionalized with citrate groups to achieve dispersion stability via electrostatic repulsion. The observed inconsistencies in toxicity profiles for similar materials have been identified to arise from difference in their dispersion stability. As discussed earlier, Wang *et al.* found that Na salts in DMEM media can lead to the disruption of electrostatic stabilization and as a result Au NPs aggregate outside the cell [42]. MWNTs are usually functionalized to achieve stable suspensions. The functionalization process involves the selective breaking of C = C bonds in the MWNT and is achieved through oxidation [45]. This results in carboxyl groups that could then be used as the platform for further reactions. In some cases, the functionalization process has been found to improve the biocompatibility of MWNTs [46]. On the other hand, some studies reported that the chemical properties of certain solubilizers and functional groups may also cause moderate toxicity and trigger immune response [27,47]. A compilation of the toxicity of various CNT solubilizers and functional moieties may be found by Firme *et al.* Apart from the toxicity arising from chemicals used in the stabilization process, Wang *et al.*, also observed significant differences between well dispersed and non-dispersed, as-prepared, and purified MWNTs in providing a stimulus for TGF- β 1 production in a bronchial epithelial cell line (BEAS-2B) as well as TGF- β 1 and PDGF-AA production in lungs of mice receiving MWNTs via oropharyngeal aspiration. Interestingly, in their studies, well-dispersed tubes induced higher IL-1 β production in a human monocytic cell line (THP-1) [48]. In contrast, carboxyl-functionalized MWNTs, although well-dispersed, induced little or no pro-fibrogenic effects in epithelial cells and limited fibrosis in the murine lung. In summary, these results indicate that the state of ENM dispersion plays an important role in immunological and physiological response.

In vitro experiments typically use cell culture plates that contain control and ENM samples in various wells. The uptake of NPs or cell viability is measured by adding desired concentrations of ENMs to each well. Often, the ENM suspension is presumed to be well-dispersed for time periods much longer than the assay time point. In reality, many ENMs sediment relatively quickly (in several minutes) and thereby increase the net dosage of ENMs perceived by the cells. Thus, the effective dosage of ENMs is highly dependent on their physicochemical stability. Cho *et al.*, measured the number of gold nanoparticles taken

up by cells in upright and inverted configurations and correlated the disparity in uptake values with the sedimentation and diffusion velocities of the nanoparticles [49]. They found that the sedimentation and diffusion velocities of the nanoparticles was independent of size, shape, density, surface coating and initial concentration of the nanoparticles. Interestingly, nanoparticles with faster sedimentation rates showed greater differences in uptake between the two (upright and inverted) configurations. Thus, the net effective dosage of ENM is dependent upon rate of aggregation. The measurement of properties such as the surface charge (via zeta potential) is thus very important in the analysis of any in vitro cytotoxicity.

Effects of defects and impurities

Defects are intrinsic to any ENM and are sometimes essential for certain application. For example, synthesis of graphene nanoribbons from MWNTs exploits the stone-wales defects via rigorous oxidation [50]. Defects in ENMs may be of several types such as: i) topological (arising from shape difference), ii) incomplete bonding defects (vacancies and dislocations), iii) dopants, and iv) grain boundaries & surface states. The defect sites may be relatively charged due to any imbalances in the crystal structure [27]. The Stone-Wales defects in MWNTs has been found to be involved in electron transfer which may generate highly reactive species leading to changes in oxidative stress. In MWNTs, there is a 1% to 3% probability of finding Stone-Wales defects randomly distributed over a length of 4 μm [51]. Fenoglio *et al.*, explored the effect of defects on the lung toxicity in their two companion papers [31, 32]. They prepared and analyzed MWNTs with various defects using Raman scattering, electron spin resonance and adsorption micro-calorimetry. Their combined physicochemical and toxicological studies showed that the reactive oxygen species scavenging property and *in vivo* toxicity are strongly defect dependent. They found that when ground, MWNTs acquired the ability to trigger inflammation and genotoxic effects, suggesting a major role of dangling bonds in both of these effects. Along the same lines, George *et al.* recently demonstrated that Ag nanoplates exhibit a higher level of toxicity compared to nanospheres and nanowires due to their increases surface reactivity due to defects [52].

As mentioned earlier, reduction in particle-size can lead to the formation of electronic states not present in the bulk phase forms. Additionally, dopants can modify these electronic properties and thereby alter the toxic response of nanomaterials. Xu *et al.* found that the cytotoxic response of metal-oxide nanoparticles (ZnO and CuO) is correlated to their conductivity [53]. They observed that Al doped ZnO NPs exhibited higher toxicity due to the increased electronic concentration arising from the dopants. Similarly, Carrero-Sanchez *et al.* observed that the intratracheal injection of N-doped MWNTs up to doses as high as 5 mg/kg did not induce any visible signs of distress on the animals, in contrast to the lethal effects of similar doses of undoped MWNTs or SWNTs [54]. More interestingly, they found that other routes of administration including oral and nasal routes also exhibited similar results for N-doped MWNTs. Such difference of toxic response was attributed to the weaker van der Waal's force among the N-doped MWNTs relative to pristine MWNTs.

Extrinsic defects such as catalyst impurities may also induce toxic response by themselves. For instance, Fe and Ni catalyst materials found in MWNTs can promote generation of oxidative species thereby contributing to the inflammatory response. This interaction triggers various redox-cycling cascades that ultimately deplete endogenous antioxidants leading to oxidative stress. When NADPH oxidase is activated inside the cell it produces superoxide species. The Fe-based catalyst impurity may then react with the superoxide species resulting in oxidative stress [27].

Effects of surface modification

Recently, many researchers have been making efforts to understand the biological effects of ENM-protein interactions. It is well known that proteins adsorb to the surface of ENMs that are entrained in a biological medium to form ENM-protein corona (ENM-PC). Any proteins with a moderate affinity for the ENM physisorb (or chemisorb) onto ENM surface to form hard corona. On the other hand, proteins with low affinity simply interact (with out any binding) with the ENM surface forming soft corona. The soft ENM-PC exchanges the adsorbed proteins with other free proteins through equilibration to form a hard corona. The dynamics of protein association/dissociation depends on the ENM's physicochemical properties such as surface charge, shape and size.

The non-specific binding between ENM and proteins can mediate the uptake of the ENM via receptor-mediated endocytosis [55]. Thus, same nanostructures may exhibit different toxic response in different media containing different protein content. MWNTs bound to the pulmonary surfactant proteins A and D lead to susceptibility to lung infection and emphysema in mice [56]. Similarly, a MWNT and α -chymotrypsin complex has been shown to inhibit enzymatic activity [57]. On the other hand, interactions of ENM and proteins are also known to enhance their biocompatibility making them nontoxic or less toxic than the pristine CNTs. Ge *et al.*, and Hu *et al.*, demonstrated that the cytotoxicity of SWNTs, graphene oxide (GO) nanosheets was mitigated when they were coated with fetal bovine serum due to their extremely high protein adsorption ability [58, 59].

Conclusions

ENMs have been proposed for various applications including drug delivery due to their unique properties. However, more understanding and research on cytotoxic and inflammatory response is needed before the viability of ENMs can be realized. To this end, it is pertinent to understand the dependence of ENM toxicity on their physicochemical properties including size, shape and surface functionality. As discussed in this review, for achieving benign drug delivery systems, the ENM should have: i) low or no inflammatory response, ii) low toxicity and iii) low tendency for protein corona formation.

In this review, we surveyed the effects of varying size and shape on the cytotoxic response of ENM. Several researchers found that 50 nm nanoparticles exhibited maximum cellular uptake. In addition to variable shapes and sizes, the inevitable presence of defects and impurities was also found to alter the toxicity response of ENMs. Further, factors such as the sedimentation rates and surface coating by proteins also have significant effects on the toxicity of ENM. The fundamental origin of ENM toxicity is highly complex since all the physicochemical properties are interrelated. As shown in Scheme 1, size, shape and defects are crucial factors in the determination of protein corona formed on an ENM while protein corona formation itself can alter the dispersion stability of the ENM system. This complex interrelation is very hard to decouple in reality and is a major roadblock in the ability to predict ENM's toxic response based on its physicochemical properties

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Scheme 1.

The interrelation amongst various physicochemical properties that determine the ultimate cytotoxic response. This complex interrelation amongst various properties may elicit different cellular (frustrated phagocytosis, cytokine production etc.) or sub-cellular (gene expression changes, changes in the production of growth factors etc.) effects resulting in the observed inflammatory and cytotoxic response.