Nanotoxicity: the growing need for *in vivo* study

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Nanotoxicology is emerging as an important subdiscipline of nanotechnology. Nanotoxicology refers to the study of the interactions of nanostructures with biological systems with an emphasis on elucidating the relationship between the physical and chemical properties (e.g. size, shape, surface chemistry, composition, and aggregation) of nanostructures with induction of toxic biological responses. In the past five years, a majority of nanotoxicity research has focused on cell culture systems; however, the data from these studies could be misleading and will require verification from animal experiments. *In vivo* systems are extremely complicated and the interactions of the nanostructures with biological components, such as proteins and cells, could lead to unique biodistribution, clearance, immune response, and metabolism. An understanding of the relationship between the physical and chemical properties of the nanostructure and their *in vivo* behavior would provide a basis for assessing toxic response and more importantly could lead to predictive models for assessing toxicity. In this review article, we describe the assumptions and challenges in the nanotoxicity field and provide a rationale for *in vivo* animal studies to assess nanotoxicity.

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Introduction

Advances in engineering nanostructures with exquisite size and shape control, elucidation of their unique properties, and demonstration of their broad applications have made nanotechnology an exciting research area [1,2,3]. Engineered nanostructures are used as probes for ultrasensitive molecular sensing and diagnostic imaging, agents for photodynamic therapy (PDT) and actuators for drug delivery, triggers for photothermal treatment, and precursors for building solar cells, electronics and light emitting diodes [1–5]. As this field transitions from academic findings to industrial products, concerns have surfaced regarding the toxicity of nanostructures [6,7,8]. Currently, a complete understanding of the size, shape, composition and aggregation-dependent interactions of nanostructures with biological systems is lacking [9] and thus it is unclear whether the exposure of humans, animals, insects and plants to engineered nanostructures could produce harmful biological responses [10]. Hence, a new subdiscipline of nanotechnology called nanotoxicology has emerged [11,12•]. There is a keen interest in nanotoxicology research because the processing of nanostructures in biological systems could lead to unpredictable effects. For example, assume that a 10-nm metallic nanostructure with an antibody-tag is injected into a patient for cancer diagnosis and assume that the toxicology of a specific physical parameter (e.g. size) of these nanostructures is well known. *In vivo*, these nanostructures could get metabolized or altered, and since it's well known that engineered nanostructures have properties that are related to their size, shape, and/or composition, the resultant effects of the metabolized/altered nanostructures on the biological system would be difficult to predict. Because of this uncertainty, in combination with the absence of a complete current understanding of the interactions of as-designed nanostructures with biological systems, regulatory agencies [9] and general public have raised questions in regards to nanotechnology-based products. The focus of this article is to provide a review of the *in vivo* activities of nanostructures and define the link between these studies and a better toxicological understanding of nanostructures. Further, we will provide a brief description of nanostructures, their interactions with cells in *in vitro* cell culture systems, and their potential molecular effects (e.g. inflammation) but these topics will not be a major focus. We refer the interested reader to a number of excellent reviews on each of these topics [2,13••,14–16,17•].

Nanostructures and the potential cause of toxicity

Chemists and material scientists are focused on engineering nanoscale materials on surfaces or in colloidal-form that contain at least one dimension smaller than 100 nm with high reproducibility and monodispersity. These new materials could have a number of possible causes of toxicity: (1) nanostructures have been demonstrated to have electronic, optical, and magnetic properties that are related to their physical dimensions, and the breakdown of these nanostructures could lead to a unique toxic effect that is difficult to predict, an example of which is provided in the introduction, [18] (2) nanostructure surfaces are involved in many catalytic and oxidative reactions. [16] If

these reactions induce cytotoxicity, the toxicity could be greater than a similar bulk material because the surface area-to-volume ratio for nanoscale material is much greater [19], and (3) some nanostructures contain metals or compounds with known toxicity and thus the breakdown of these materials could elicit similar toxic responses to the components themselves. In Table 1, we highlight the applications, concerns, and biological/mechanistic studies for some of the most commonly used nanostructures.

Current assumptions about nanostructures

Currently, there is a common assumption that the small size of nanostructures allows them to easily enter tissues, cells, organelles, and functional biomolecular structures (i.e. DNA, ribosomes) since the actual physical size of an engineered nanostructure is similar to many biological molecules (e.g. antibodies, proteins) and structures (e.g. viruses). A corollary is that the entry of the nanostructures into vital biological systems could cause damage, which could subsequently cause harm to human health. However, a number of recent studies have demonstrated that despite the size of the nanostructures they do not freely go into all biological systems but are instead governed by the functional molecules added to their surfaces. For example, citrate-stabilized gold nanostructures entered the mammalian cells but were not able to enter the cytoplasm or nucleus [20]; whereas one can engineer the nanostructure's surface chemistry for access to the nucleus or mitochondria [21]. A number of *in vivo* studies have also shown that nanostructures have difficulty entering the brain, which is protected by the blood–brain barrier, unless aided by tailored surface functionalization. Researchers can now engineer nanostructures to direct the intracellular or *in vivo* biodistribution [21–23] but the final metabolic fate is still unknown, and strategies for avoiding secondary unintentional behaviors are lacking. Overall the relationship between size, shape, and surface chemistry of nanostructures [24] and their correlation to intracellular and *in vivo* bio-distribution is unknown. By contrast, pharmaceutical strategies have developed this sort of relationship for a number of drugs and carriers and thus, they have created predictive categorization which will need to be emulated with nanostructures. Systematically, one cannot predict the movements and location of nanostructures after intracellular or *in vivo* exposure based on nanostructure properties at this time, and such studies must be done before one can assess the toxicity of nanostructures in a systematic format.

What can one learn from *in vivo* studies

A systematic and thorough quantitative analysis of the pharmacokinetics (absorption, distribution, metabolism, and excretion; PK) of nanostructures can lead to improvements in design of nanostructures for diagnostic and therapeutic applications, a better understanding of nanostructures non-specificity toward tissues and cell types, and assessments of basic distribution and clearance that serve as the basis in determining their toxicity and future investigative directions. PK gives the quantitative *in vivo* conditions under which the dose achieves or causes any observed toxic effects. Toxicity to specific cell types can be qualified by PK in that the time and concentrations to which they will be exposed can be determined. Residence time and accumulation locations of the dose and metabolites can be the difference between avoiding and experiencing toxic responses.

The overall behavior of nanostructures could be summed as follow: (1) nanostructures can enter the body via six principle routes: intra venous, dermal, subcutaneous, inhalation, intraperitoneal, and oral [25], (2) absorption can occur where the nanostructures first interact with biological components (proteins, cells), (3) afterward they can distribute to various organs in the body and may remain the same structurally, be modified, or metabolized [18], (4) they enter the cells of the organ and reside in the cells for an unknown amount of time before leaving to

Table 1

Survey of prevalent nanostructure classes, examples of their proven applications, the current status of their *in vivo* characterization, and the biological areas of concern involved

Nanostructure	Application (example)	Concerns	Mechanistic areas of interest	References
Metal nanoparticles	Contrast agents; drug delivery	Element specific toxicity; reactive oxygen species	Excretion	[2,19,40,41]
Nanoshells	Hyperthermia therapy	None demonstrated	Excretion	[2,3,54]
Fullerenes	Vaccine adjuncts; hyperthermia therapy	Antibody generation	Immunotoxicity	[2,11,13••,17•]
Quantum dots	Fluorescent contrast agent	Metabolism	Intracellular/ organ redistribution; excretion	[1,2,26•,27,39,43••,50]
Polymer nanoparticles	Drug delivery; therapeutics	Unknown	Metabolism; immunotoxicity; complement activation	[3,13••,19,32••,46•]
Dendrimer	Guest delivery of drug/radiolabel dose	Metabolic path	Surface chemistry and elemental effects; complement activation	[2,14,38]
Liposome	Drug delivery; contrast agent vehicle	Hypersensitivity reactions	Complement activation	[2,13••]

move to other organs or to be excreted. Of note, excretion could also occur between steps 3 and 4. In this section of the review, our aim will be to provide context but not to draw any major conclusions since there are limited studies on the PK of engineered nanostructures.

Many people can get exposed to nanostructures in a variety of manners such as researchers manufacturing the nanostructures, patients injected with nanostructures, or people using products containing nanostructures. In all cases, there will be unique routes of exposure that will dictate the specific fate of the nanostructures. Most of the recent studies in this area have focused on the absorption of the nanostructures via inhalation or dermal exposure. A number of these studies have focused on traditional toxic materials such as asbestos and carbon black, which contains materials with a large size distribution (some of which are nanometer in scale) [8,11]. For the recently engineered nanostructures with well-defined sizes, there are limited studies. It has been discovered that quantum dots (QDs) in porcine skin show variability in absorption kinetics because of coating chemistry, with periodic variation in cellular uptake [26•]. Orally administered nanostructures do not seem to be absorbed and are recovered in feces [22,27]. These studies suggest the importance of exposure route and physical properties of the nanostructures on absorption behavior but again, there is not enough data to make a general conclusion.

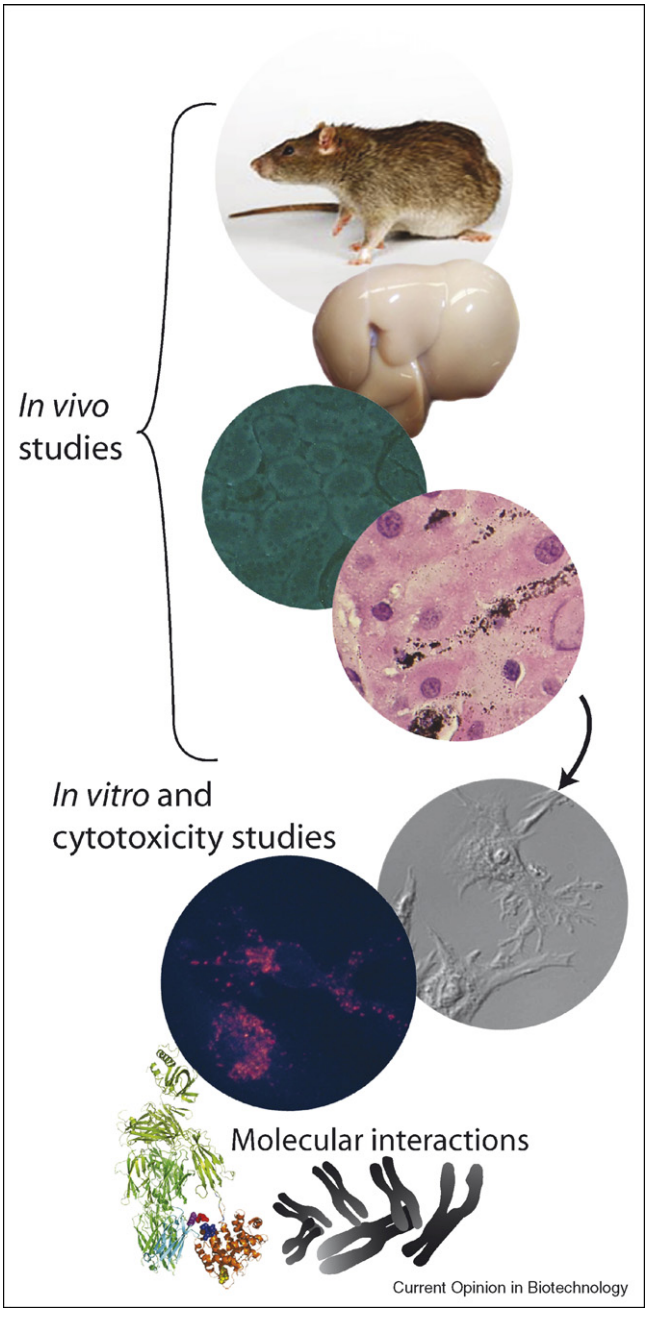
During the absorption process, the nanostructures may interact with many biological components. The most obvious stems from interactions with proteins, a process known as opsonization. The protein corona, a complex competition between adsorbing proteins and the equilibrium they reach on these highly curved surfaces [28•], will play a central role in determining what surface is actually presented to cells which take the nanostructure up or/and activate signaling pathways [29]. Further, there is a need to understand the binding kinetics involved in the interactions between nanostructures and the protein(s) on or in cells. Conducting these studies requires a combination of analytical techniques, which are outlined in ref [29,30••]. In addition, conformational changes of proteins adsorbed onto nanostructure surfaces could alter the function of the protein [28•,31] and would affect the fate of the nanostructures. Careful cataloguing of what serum proteins interact with nanostructures, and how physico-chemical properties alter the amount, composition and possible activation of the adsorbed proteins will be a large step towards anticipating what absorption and distribution kinetics will be observed. Many of the subset of serum proteins that interacts with nanostructures such as complement [32••] and immunoglobulins are immunoactive. Thus, an interesting avenue of future research is the broadening of PK investigation to cover immune components more carefully. [13••] A correlation of nanostructure–protein interaction [32••] with *in vivo* PK data permits the assembly of a structure–activity relationship; this represents an important next step for evaluating nanotoxicity.

After absorption, nanostructures distribute to various organs, tissues, and cells. Only a few recent studies have focused on *in vivo* biodistribution of engineered nanostructures as it relate to the nanostructure's physical parameters. In these studies, the key is to quantitatively map the location of the nanostructures at different time points and at different dose. Thus far, we cannot make a general conclusion as to how size, shape, aggregation and surface chemistry affect nanostructure bio-distribution. In studies with QDs and single-walled carbon nanotubes (SWCNT), it was discovered that a high dose of the QDs is sequestered in the liver and the percentage of the QD or SWCNT dose sequestered is dependent upon the surface modification [27,33•,34]. Other organs that can take up nanostructures include the spleen, lymph node, and bone marrow. All of these organs contain large concentrations of macrophages, which are part of the reticuloendothelial system (RES), and thus we can conclude that nanostructures are taken up by phagocytic cells. The RES system, now called the mononuclear phagocyte system (MPS), is part of the immune system and consists of a collection of monocytes and macrophages involved in the uptake and metabolism of foreign molecules and particulates [35]. Nanostructures that are coated with the polymer polyethylene glycol have avoided RES uptake [36]. In another example, MWCNT were shown to evade the RES when their surface is coated with ammonium/chelator functional groups [37•] but were taken up when coated with taurine [22]. Aside from the surface chemistry, the core nanostructure could also impact the bio-distribution behavior. It was shown that dendrimers with gold incorporated into the structure elicit different organ distribution [38]. A question remains: how much does each of the chemical and physical parameters contribute to the distribution of the nanostructures?

Once distributed and sequestered in cells, how core nanostructures are metabolically processed is still not fully addressed. Polymer-based nanostructures and super paramagnetic iron oxide nanostructures for MRI contrast agents are shown to degrade, but QDs, fullerenes, silica nanoparticles are examples of nanostructures without clear indication of degradation *in vivo* [27,37•,39] For example, Fischer *et al.* [33•] and Ballou *et al.* [39] show that the core ZnS-capped CdSe QDs remain intact and fluorescent *in vivo* after one month; however, neither study analyzed the metabolism of the organic coating on the nanostructure's surface. The breakdown of the nanostructures could elicit unique molecular responses that are not predictable and thus, the understanding and cataloging of what, when, and how much nanostructures degrade is extremely important.

In regards to excretion, there are many possible routes. These routes include the kidney [37•] or the liver/bile duct [40,41]. Hydroxl functionalized SWCNT dosed intraperitoneally accumulate in the liver and kidneys, and are excreted in the urine in 18 days, [42]; whereas, ammonium functionalized SWCNT dosed *intravenously* showed no liver uptake and much faster renal excretion [37•]. For nanostructures such as QDs, two initial studies showed they do not excrete and remain intact *in vivo* [27,33•]. This, however, has very recently been demonstrated to be size and surface chemistry dependent. Reddy et al showed that QDs smaller than 5.5 nm in diameter, which are cysteine coated, are excreted in the urine [43••]. If not excreted in this manner, how long they reside and what happens to their long-term behavior *in vivo* remains unclear.

Figure 1



Progression of studies required to fully assessing the impact of physicochemical nanostructure properties. Studies in animal models will identify the organs of interest, in turn leading to identifying the best cell types for studies, and to focused studies on how these cells molecularly respond to the nanostructures. A number of the images were adapted with reprint permission from the Public Division of the Massachusetts Medical Association [50].

We present an overall rationale for conducting PK studies as a first route for assessing nanotoxicity. The information obtained from such studies identifies the cells that take them up, which could logically lead to more focused studies (see Figure 1). For example, since the liver is involved in nanostructure uptake, biochemical indicators of liver stress were examined in response to multiwalled CNT showed no negative effects after 28 days despite accumulation in that organ [22]. Inflammation in response to nanostructures has been observed, though, with gene expression analysis of rat lungs, showing upregulation of transcription factors associated with cell responses to oxidative stress [44]. QDs have also activated astrocytes in the brain upon direct injection, depending on surface functionalization [45], and nanostructure size has been shown to influence the ability to produce CD8 and CD4 type 1 T cell responses, with those between 40 and 50 nm at a maximum effect [46•,47]. These specific studies will identify the organ that could potentially be stressed by exposure to nanostructures and will provide a molecular basis of the stress. If one can associate these responses to specific types, surface chemistry, size, shape, aggregation and composition, then we would be able to correlate the toxic responses to the properties of the nanostructures.

A case for *in vivo* studies

Once the specific organs and cells are identified, further focused studies using *in vitro* models (e.g. cell culture) would complement the *in vivo* studies. Currently, most studies in this area of research utilize cell culture models, in which the results could only be considered as exploratory. The combined results from multiple studies of different cells *in vitro* cannot be assumed to capture the same behavior as the same cells arranged *in situ* in an organ. The lack of correlation between *in vivo* and *in vitro* effects has recently been demonstrated [48], indicating that *in vitro* experiments must have *in vivo* validation in order to be useful. Intercellular effects such as macrophage and dendritic cell recruitment require that the cells and their signaling occur in its natural state. Additionally, the involvement and influence of transport mechanisms such as lymph, blood, and bile, cannot be incorporated into *in vitro* studies. Since the nanostruc-

tures are being recognized by the body's natural defenses against foreign matter, it is important to investigate the immune system's involvement in its entirety. It is also necessary to determine which cells to expose the materials to. For example, early *in vitro* work on QD hypothesized that hepatocytes would be involved in QD metabolism because hepatocytes are involve in metal detoxification [23], but more insight gained through recent PK [27,33•,39], has shown that Kupffer cells, resident liver macrophages, are responsible for the considerable liver uptake observed in most of the current intra venous nanostructure distribution experiments. More recent *in vitro* work has continued in this approach of comparing *in vitro* work with corresponding *in vivo* studies and exposes a need for better agreement between the two [48,49••].

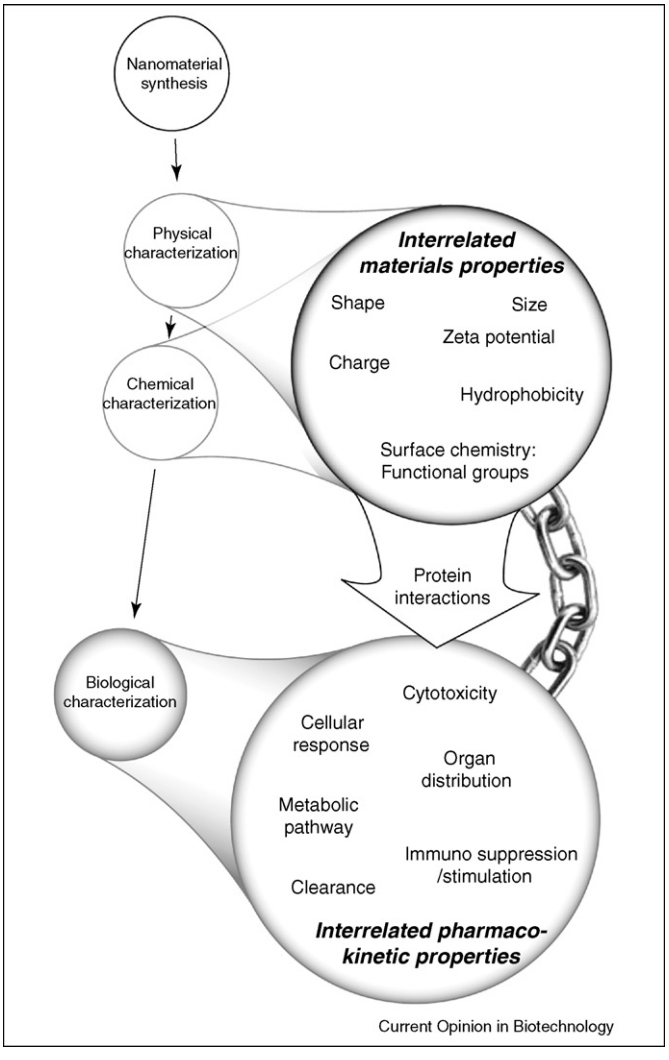
Challenges

In vivo studies of nanostructures provide new challenges to how pharmaceutical sciences traditionally conduct PK research. Detection strategies must be capable of quantifying all of the major parts of a nanostructure in tissues and organs since many modern nanostructures are engineered with multiple components. For example, bio-targeted nanostructures are typically designed with an inorganic core that is coated with stabilizing molecules and/or coated with biological molecules. Traditional radiolabeling of the organic surface molecule coating on the core nanostructure is easily achieved, but the PK data using this labeling scheme can be misleading. The *in vivo* metabolic trajectory of the labeled compound may differ from the core nanostructure. This shows that techniques must be flexible and tailored to the specific nanostructure being investigated, and that the investigators be cognoscente of the correlation between chosen metric and actual interpreted quantity. Multi-indicator techniques, in which each of the three components listed above would incorporate distinct markers, would provide a complete picture of metabolic processes but this has not been applied to nanostructures.

Another challenge is the variability in manufacturing methods, associated raw materials, and reaction scaling necessary to produce adequate volumes of uniform nanostructures. Consistent particle properties and a classification scheme to support it are necessary to be able to make cross comparisons between the results obtained from different research groups [51•]. A recent example is the obvious shape difference between the ZnS-capped CdSe QDs used by Gopee *et al.* [52] which are 'nail shaped' and those used in our work, which were spherical [33•]. Consistency on this front is needed before further work can be done to determine the magnitude of inter-lab, species, and nanostructure variation.

Once the detection and synthetic challenges are overcome, significant infill of data is required to construct an accurate picture of what variables influence nanostructure PK and whether toxicity will be observed. Systematic studies that investigate a range of chronic and acute exposure doses of nanostructures with different physical or chemical variables such as size, charge, and hydrophobicity have not been fully conducted. Even more absent, are studies where more than one of these nanostructure properties are varied in conjunction with one another to determine associative properties. How both of these aspects impact distribution would also benefit from increased detail of data relating to distribution location. Increasing the resolution of *in vivo* distribution to the cellular level will enable more effective correlation between nanostructure properties and *in vitro* cytotoxicity and metabolism data.

Figure 2



The nanostructure physico-chemical property relationship to *in vivo* responses. Each property (as listed) of the nanostructure could influence the biological response. Studies must fully characterize the nanostructure properties before proper interpretation of biological results since they are interrelated.

Another major challenge with nanostructure *in vivo* studies is understanding how proteins interact with nanostructure surfaces (reviewed recently in this journal [53]). In order to qualify observed *in vivo* results, an understanding of the nanostructure protein-interface is vital, since this can potentially dictate behavior *in vivo* as illustrated in Figure 2.

Conclusions

In this review article, we provide a general discussion on the need for *in vivo* studies to accurately assess nanotoxicity. This subfield of nanotechnology is important for the advancement of nanotechnology in a wide-array of applications. Furthermore, studies in this area could lead to the required information to make responsible regulatory decisions. Thus far, most reported nanotoxicity studies have focused on *in vitro* cell culture studies, however, data obtained from such studies may not correspond to *in vivo* results. In the future, a full *in vivo* 'life cycle' characterization framework needs to be formulated wherein systematic evaluation of the size, shape, and surface chemistry of nanostructures, and their correlation to *in vivo* behavior, is obtained. This permits the mapping of the fate, kinetics, clearance, metabolism, protein coating, immune response and toxicity of nanostructures to the nanostructure's physical properties. This would allow the development of predictive models of nanostructure toxicity.

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