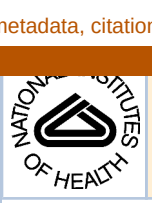

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Effects of nanomaterial physicochemical properties on *in vivo* toxicity

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Abstract

It is well recognized that physical and chemical properties of materials can alter dramatically at nanoscopic scale, and the growing use of nanotechnologies requires careful assessment of unexpected toxicities and biological interactions. However, most *in vivo* toxicity concerns focus primarily on pulmonary, oral, and dermal exposure to ultrafine particles. As nanomaterials expand as therapeutics and as diagnostic tools, parenteral administration of engineered nanomaterials should also be recognized as a critical aspect for toxicity consideration. Due to the complex nature of nanomaterials, conflicting studies have led to different views of their safety. Here, the physicochemical properties of four representative nanomaterials (dendrimers, carbon nanotubes, quantum dots, and gold nanoparticles) as it relates to their toxicity after systemic exposure is discussed.

Keywords

nanomaterial; toxicity; *in vivo*; dendrimer; carbon nanotube; quantum dot; gold nanoparticle; analysis

1. Introduction

Research involving the use of different types and designs of nanomaterials continues to evolve with the growth of nanotechnology for *in vivo* applications in such fields as drug delivery, medical imaging, diagnostics, and engineering technology [1-4]. Currently, several therapeutics are approved for use or are in clinical trials and it is expected that nanotechnology will be utilized in many more commercial products in the near future [4,5].

The majority of nanotoxicity studies have focused on health effects of exposure to ultrafine (unintentionally produced) particles by inhalation, contact through skin, or ingestion [1,6-9]. These studies primarily focus on local effects (e.g. lung toxicity after particle inhalation). With a multitude of opportunities for nanomaterial use in pharmaceutical and medical applications, a thorough understanding of associated systemic toxicity is critical. Characterization of *in vivo* toxicity has been a daunting task as nanomaterials are quite complex and conflicting

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studies have led to different views of their use and safety. This makes it difficult to evaluate, generalize, and predict important aspects of toxicity [10]. Presented in this review are current studies evaluating the systemic toxicity of four representative nanomaterials (dendrimers, carbon nanotubes, quantum dots, and gold nanoparticles) as it relates to nanomaterial properties and design.

1.1. Mechanisms of nanomaterial toxicity

Nanomaterial toxicity can occur through several different mechanisms in the body. A summary of the major forms of *in vivo* nanomaterial toxicity is displayed in Fig. 1. As summarized by Lanone et al., the main molecular mechanism of *in vivo* nanotoxicity is the induction of oxidative stress by free radical formation [3]. In excess, free radicals cause damage to biological components through oxidation of lipids, proteins, and DNA. Oxidative stress may have a role in the induction or the enhancement of inflammation through upregulation of redox sensitive transcription factors (e.g. NF-κB), activator protein-1, and kinases involved in inflammation [3,11,12]. Free radicals can originate from several sources including phagocytic cell response to foreign material, insufficient amounts of anti-oxidants, presence of transition metals, environmental factors, and, as focused in this review, physicochemical properties of some nanomaterials [3]. Slow clearance and tissue accumulation (storage) of potential free radical producing nanomaterials as well as prevalence of numerous phagocytic cells in the organs of the reticuloendothelial system (RES) makes organs such as the liver and spleen main targets of oxidative stress. Additionally, organs of high blood flow that are exposed to nanomaterials, such as the kidneys and lungs, can also be affected.

Intracellularly, nanomaterials may interact with cellular components, disrupt or alter cell function, or create reactive oxygen species (ROS). Interactions of nanomaterials with the mitochondria and cell nucleus are being considered as main sources of toxicity. As reviewed by Unfried et al., nanomaterials such as silver-coated gold nanoparticles, fullerenes, block copolymer micelles, and carbon nanotubes may be capable of localizing to mitochondria and inducing apoptosis and ROS formation and that nanomaterial-induced nuclear DNA damage, cell-cycle arrest, mutagenesis, and apoptosis is a possible source of toxicity [13]. Although still under debate, nanomaterials may be involved in the upregulation of NADPH oxidase and xanthine oxidase, which are free radical sources in macrophages and neutrophils [3].

Other mechanisms of toxicity from nanomaterials should be considered since nanomaterials immediately interact with their surrounding environment. When introduced or absorbed into the systemic circulation, interaction with blood components can lead to hemolysis and thrombosis. Additionally, nanomaterial interactions with the immune system have been known to increase immunotoxicity as reviewed by Dobrovolskaia et al. [5]. In the liver, further metabolic modification of nanomaterials, e.g. by cytochrome P450, may result in hepatotoxicity by reactive intermediates [3].

1.2. Nanomaterial design considerations

Nanomaterials have unique properties and characteristics relative to bulk materials (e.g. high surface area to volume ratio) that may endow them with unique mechanisms of toxicity from xenobiotics. In particular, toxicity has been thought to originate from nanomaterial size and surface area, composition, and shape as reviewed by Lanone et al. [3].

Size plays a role in how the body responds to, distributes and eliminates materials [10,14]. Particle size can also affect the mode of endocytosis, cellular uptake, and the efficiency of particle processing in the endocytic pathway [3,15]. *In vitro* studies of non-phagocytic cellular uptake of latex spheres have demonstrated slower uptake and processing of large spheres (>200 nm) relative to small ones (50 and 100 nm) [15]. More importantly, decreasing size also leads

to an exponential increase in surface area relative to volume, thus making the nanomaterial surface more reactive on itself (aggregation) and to its surrounding environment (biological components). Increased uptake into certain tissues may lead to accumulation, where they may interfere with critical biological functions [3,7].

Chemical composition at the surface of nanomaterials will largely define their chemical interactions since the surface is in direct contact with the body whereas the limited bulk volume is hidden [10]. Therefore, nanomaterials behave quite differently than bulk materials. Many nanomaterials are functionalized on the surface to increase blood circulation, make them more biocompatible, and for targeted therapy. While functionalization has shown promise in many applications, functional groups added to the surface can potentially interact with biological components, alter biological function, and allow passage of nanomaterials that would not normally be taken up by certain cells.

Degradability of the material is an important component of acute and long-term toxicity. Non-degradable nanomaterials can accumulate in organs and also intracellularly where they can cause detrimental effects to the cell, similar to that of lysosomal storage diseases [2]. In contrast, biodegradable nanomaterials can lead to unpredicted toxicity due to unexpected toxic degradation products [6]. Nanomaterials may contain transition metals (e.g. quantum dots) or other compounds with known toxicity that are “masked” for instance by functionalization. Degradation of this material may release toxins to the biological milieu, leading to free radical formation and resulting in cellular damage [3,6].

As elicudated from the effects of asbestos fibers in the lungs and studies of intratracheal instillation, nanomaterials may be capable of evading macrophage uptake and RES clearance [8,16-18]. Although known that slow dissolution of particles can overwhelm macrophage-mediated clearance resulting in particle persistence, the shape of nanomaterial may also play a role in effective clearance. Recent investigations by Champion et al. have described macrophage interaction with different shaped nanomaterials [19]. They discovered that failure to create the required actin-driven movement of the macrophage membrane results in the macrophage spreading onto the material rather than internalization. Therefore, complete phagocytosis is based on the point of macrophage contact, with less internalization with rod-like materials relative to spherical materials. Though this study was specific to alveolar macrophages, similar results may be applicable to other tissues.

2. *In vivo* toxicity of nanomaterials

The uniqueness of each type of nanomaterial being explored for medical applications makes generalization of nanomaterial toxicity rather complicated. To elucidate causes of toxicity, current research evaluating the distribution and toxicity of nanomaterials usually focuses on one aspect of the material properties at a time (e.g. effect of altering surface charge). Current research on the effects of nanomaterial properties of selected types of nanomaterials is discussed in this section with the primary nanomaterial toxicity concerns summarized in Table 1.

2.1. Dendrimers

Traditional polymer drug delivery systems utilize linear, random-coil polymers such as polyethylene glycol, *N*-(2-hydroxypropyl) methacrylaminde copolymers, poly (ethyleneimine), poly(lactic acid), poly(glycolic acid) and their copolymers, dextrin (α-1, 4 polyglucose), hyaluronic acid, chitosans, poly(glutamic acid), and poly(aspartamides) [20, 21]. The major obstacle using these polymeric materials in biomedical and pharmaceutical applications is the heterogenic nature of these polymers. The conventional polymerization methods for these linear polymers usually result in polymers with different chain lengths

(molecular weights), different chemical structures, and polydispersity. Therefore, these polymers are not reproducible batch to batch, which makes it difficult to define their *in vivo* behaviors (e.g. biodistribution, pharmacokinetics, stability, toxicity) using validated techniques. Low drug carrying capacity of these linear polymers and variable drug release kinetics *in vivo* all contribute to the lack of improved therapeutic index of the loaded drug.

Dendrimers, also called arboroles or cascade polymers, are well-defined, highly branched macromolecules with precisely controlled chemical structure and low polydispersity (Fig. 2). Dendrimers are synthesized in a stepwise process either by a “divergent method” or “convergent method”. In the divergent method, synthesis starts with a multifunctional core followed by repeated addition of monomers and increase in molecular weight with exponential increase in surface termini. By contrast, the convergent method starts from the dendrimer surface and proceeds inward to a multivalent core where the dendrimer segments are joined together. Dendrimer branching offers advantages such as nano-size spherical structures (for high generations), low viscosity compared to equivalent molecular weight linear polymers, narrow polydispersity, and high density of surface functionalities [22]. Over the past three decades, dendrimers such as polyamidoamine (PAMAM, i.e. Starburst™) [23,24], poly (propylenemine) (PPI) [25,26], triazine [27,28], and poly-L-lysine (PLL) [29-31] have been used widely as vehicles for drug or gene delivery. However, the *in vivo* toxicity of dendrimers has not been systematically investigated [32]. This review mainly focuses on PAMAM dendrimers since they are one of the most studied dendrimers.

Depending on the dendrimer type and generation, they carry a positive, neutral, or negative charge on the surface. Once introduced into the systemic circulation, positively charged dendrimers and cationic macromolecules have been found to interact with blood components, destabilize cell membranes, and cause cell lysis [33-35]. Roberts et al. studied the toxicity of cationic PAMAM Starburst® in mice [24]. Cationic dendrimers of generation 3 (G3, MW 5147, diameter 31 Å, 24 amine termini), generation 5 (G5, MW 21563, diameter 53 Å, 96 amine termini), and generation 7 (G7, MW 87227, diameter 80 Å, 768 amine termini) were given i.p. with doses ranging from 0.026 to 45 mg/kg either as a single dose or once a week for 10 weeks. The *in vivo* toxicity was evaluated by behavioral abnormalities 2 h post injection and by body weight changes up to 6 months. No significant behavioral abnormality or weight loss was observed through the study. However, one out of five mice died at about 24 h after dendrimer injection in the G7 group with highest dose of 45 mg/kg. These studies suggest that low generation cationic dendrimers ($G < 7$) even at high doses do not cause adverse effects. However, Heiden et al. found cationic PAMAM dendrimers (G4, amine termini) were toxic to zebrafish embryos, and the toxicity was dose and exposure duration dependent [36]. A mortality of 100% was observed by 24 h post fertilization when the dendrimer concentration exceeded 20 μM. In the same study by contrast, anionic PAMAM dendrimers (G3.5, carboxylic acid termini) were not toxic and showed no signs of attenuating embryo development. G4 dendrimers conjugated to Arg-Gly-Asp (RGD) were much less toxic compared to unconjugated G4 dendrimers. Malik et al. also found that 95 mg/kg daily doses of anionic PAMAM dendrimers (G3.5, carboxylic acid termini) administered i.p. led to no mortalities and no weight change in B16F10 tumor bearing mice [37]. Overall, these studies indicated that dendrimer toxicity is dose and generation dependent with higher dose and higher generation dendrimers leading to more toxicity *in vivo*. The toxicity profiles also depend greatly on the surface charge of the dendrimers, that is, anionic dendrimers are less toxic compared to cationic dendrimers.

Hematological toxicity is one of the most important concerns for dendrimers given intravenously. Cationic dendrimers such as -NH_2 PAMAM have shown concentration and generation dependent hemolysis and induced morphology changes in red blood cells after 1 h incubation at a low concentration of 10 μg/mL; whereas, anionic dendrimers were not hemolytic at concentrations up to 2 mg/mL [23]. Agashe et al. examined the hematological

toxicities of generation 5 PPI dendrimer (PPI-5.0G), t-BOC-protected glycine-coated dendrimer (DBG), t-BOC-protected phenylalanine-coated dendrimer (DBPA), mannose-coated dendrimer (M-PPI) and lactose-coated dendrimer (L-PPI) [25]. Dendrimers were administered i.v. into male albino rats (equivalent to 1-4 mg of each polymer), and after 7 days, blood samples were collected and analyzed to determine hematological parameters such as white blood corpuscles (WBCs), red blood cells (RBCs), hemoglobin (Hb), haematocrit (HCT) and mean corpuscular hemoglobin (MCH). Compared to the control group, there were significantly higher WBC counts and lower RBC, HCT, Hb, and MCH values for the PPI-5.0G group, probably due to the polycationic nature of the dendrimers. Surface modified dendrimers (DBG, DBPA, M-PPI and L-PPI) showed no difference in terms of hematological parameters compared to the control group.

Immunogenicity is the ability of antigens to induce humoral or cell-mediated immune responses. Immunogenicity should be avoided if the immune system is not the intended target of the dendrimer drug delivery system. The immunogenicity of PAMAM (G3, G5 and G7) dendrimers at a dose of 5×10^{-5} mmol was studied in rabbits, and no evidence of immunogenicity was found 10 days after i.p. injection by the immunoprecipitation assay or the Ouchterlony double diffusion assay [24]. Five PPI-based dendrimers (PPI-5.0G, DBG, DBPA, M-PPI and L-PPI) were injected intramuscularly into Balb/C mice at dose levels of 1-4 mg of dendrimer [25]. Blood samples were collected after 21 days and antibody (IgG) titre was tested by ELISA. The antibody (IgG) level in blood was non-detectable, which indicated no sign of humoral immune response triggered by the dendrimers. However, these studies only tested short-term immunogenicity. The *in vivo* studies of cell-medicated immune response and complement activation have not been reported though a G5 PAMAM dendrimer has been seen to induce strong complement activation *in vitro* [38].

Nephrotoxicity and hepatotoxicity have been reported consistent with biodistribution studies that revealed high kidney and liver accumulation for G3 PAMAM dendrimers (i.p.) [24], G3 and G4 PAMAM dendrimers (i.v. or i.p.) [23], biotinylated-PAMAM dendrimers (G0-4) (i.v.) [39], and PEG-polyester dendritic hybrids (i.v.) [40]. Roberts et al. noticed liver cell vacuolization of the cytoplasm in a 6-month toxicity study group after administration of G7 PAMAM dendrimers by i.p. route once a week for 10 weeks [24]. A third generation melamine dendrimer (MW 8067, 24 amine termini) was evaluated for *in vivo* acute (single dose, 48 h) and subchronic (three i.p. injections every 3 weeks over 6 weeks) toxicities [41]. The acute study indicated the lethal dose was 160 mg/kg, giving rise to 100% mortality 6-12 h post injection. In both acute and subchronic studies, there was no significant renal damage in all dose ranges as determined by blood urea nitrogen levels. Hepatic function was normal at up to 10 mg/kg dendrimer as evaluated by changes in serum alanine transaminase (ALT) activity. However, a significant increase in ALT activity noticed for both acute and subchronic groups at the 40 mg/kg dose level, and the histopathological investigation showed extensive liver necrosis.

Dendrimer modification with chemically inert polyethylene glycol (PEG) or fatty acids is one of the most attractive ways to reduce dendrimer toxicities *in vitro* [35,42-44]. PEG-polyester dendritic hybrids (3-arm PEG star and G2 polyester dendrons, MW 23,500 Da) were injected into mice as an i.v. bolus dose of 1.3 g/kg [40]. Mice survived after 24 h, and no changes of organ pathology were observed in the liver, lungs, heart, kidney, or intestine. PEGylated melamine dendrimer (G3, PEG 2000 termini) was administered at doses of 2.56 g/kg i.p. and 1.28 g/kg i.v. into C3H mice, and all mice survived after 24 h and 48 h after i.p. and i.v. administration, respectively, with no liver or kidney toxicity noted [45].

The non-biodegradability of PAMAM/PPI-type dendrimers is of major concern for their medical use. Biodistribution studies of magnetic resonance imaging (MRI) contrast agents

based on PAMAM/PPI dendrimer-gadolinium (Gd) chelates have been reported. Lower generations of PAMAM-Gd (diameter < 10 nm) were excreted via kidney and the renal elimination rate decreased with increasing dendrimer size. Minimal renal excretion and high RES uptake were observed when PAMAM-Gd size exceeded 10 nm [46-49]. It was also noticed that only less than 15% of the injected PAMAM-Gd agents (PAMAM G4, G5, and G6) were excreted within 2 days [49]. Dendrimer core structure also influences the biodistribution of the dendrimer MRI agents. Compared to the PAMAM-Gd, a less hydrophilic PPI diaminobutane core contributes to the dramatic increase in liver accumulation [50]. Although PEGylation and other surface modification schemes may reduce the potential of harmful biological interactions, the dendrimer core material must be biodegradable or readily eliminated by the renal or hepatic system. Otherwise, the long-term toxicity is difficult to predict. The amide core of PAMAM and PPI type dendrimers cannot undergo hydrolysis or enzymatic degradation. Newer dendrimers where the core is structured from amino acids, oligonucleotides, or polyesters may overcome these toxicity concerns [51-56].

In conclusion, *in vivo* dendrimer toxicity profiles are closely related to the chemical structure of dendrimer; size and generation; exposure duration; biodistribution; and the rate, location, and mechanism of metabolism. Toxicity of dendrimers is influenced by the nature of the terminal groups. Full generation PAMAM dendrimers with cationic amine terminal groups are more toxic than half-generation dendrimers with anionic carboxylic acid terminal groups. High generations and high doses of cationic dendrimers usually lead to greater *in vivo* toxicity. Surface modification with PEG or fatty acids improves dendrimer biocompatibility. In order to design a more safe and non-toxic dendrimer drug delivery system, larger and more systematic toxicity studies are required.

2.2. Carbon nanotubes

Carbon nanotubes (CNT) are a class of fullerenes consisting of a sheet of graphene arranged into small cylindrical structures (Fig. 2). There at two types of CNT: single-walled (SWCNT) and multi-walled (MWCNT). SWCNT are a single tube of graphene with a diameter ranging from 0.4-3.0 nm and a length up to 1 μm [57,58]. MWCNT are multiple layers of tubes stacking within each other with an overall diameter of 1.4-100 nm and can be up to several microns long [58]. In the last few years, both SWCNT and MWCNT have been utilized as nanocarriers for parenteral drug and gene delivery and recently as targeted cancer treatments [3,59]. The safety of CNT is still in debate due to the lack of systematic and complete toxicity evaluation [60]. Recently, studies have indicated that CNT responses are similar to the carcinogenic responses of asbestos fibers when injected into the peritoneal cavity [61,62]. Due to the high aspect ratio of CNT (>100), it is expected that CNT would behave as biopersistent fibers *in vivo*. Studies have implicated size (aggregation), CNT length, and manufacturing impurities as sources for potential toxicity *in vivo* and will be discussed herein.

The size of aggregated CNT is thought to be a primary concern for toxicity. Pristine (non-functionalized) CNT are inherently hydrophobic; therefore, aggregation is expected and observed *in vivo.* For injection, pristine CNT are suspended in biocompatible surfactants such as Tween 80 or Pluronic F108 [63,64]. Several studies have been conducted on the *in vivo* distribution of i.v. injected pristine SWCNT. Primarily, accumulation of CNT was determined to be in the liver, but also in the spleen and lungs. No acute toxicity was observed in any tissues up to 24 h [63-65]. Accumulation in the liver was suggested to be due to rapid surfactant displacement followed by opsonization of serum proteins [63]. Yang et al. followed up distribution studies by looking at serum biomarkers of damage. Additionally, they looked at markers for oxidative stress (glutathione and malondialdehyde) in liver and lung samples post dose [64]. Elevated levels of lactose dehydrogenase and alanine aminotransferase were concluded to be due to hepatic injury from accumulation in the liver. They also found an

increase in malondialdehyde and a decrease in glutathione in liver and lung samples (at 1.0 mg/mouse), which was indicative of increased levels of oxidative stress. Although no acute toxicity was determined histologically up to 90 days post dose, biomarkers indicating hepatic injury due to oxidative stress should be further investigated [64]. Studies conducted longer than 90 days may exhibit more toxicity damage over time, similar to onset of damage from asbestos fibers in lungs, which could take years. ROS formation and cytotoxic effects induced by aggregates and accumulation have been observed in other studies of CNT [66,67]. A study by Faczek et al. compared pristine SWCNT and MWCNT compatibility in muscle implants [68]. They determined that both types of CNT formed aggregates when implanted in rat skeletal muscle; however, MWCNT produced larger aggregates that continued to get larger over the 90-day study. SWCNT agglomerates remained the same size throughout the study and underwent phagocytosis with subsequent drainage to the lymph nodes. MWCNT, on the other hand, did not translocate to the lymph and remained accumulated in the muscle tissue [68]. Improper phagocytosis may be due to the large diameter of the MWCNT based on aggregation. Reduction of CNT accumulation and aggregation is achieved by functionalization [69-71]. Rapid distribution and renal clearance from almost all tissues is observed 1-3 days after injection of functionalized CNT, whereas pristine CNT were determined at high levels in RES tissues up to 1 month after injection [65]. Even though functionalization of carbon nanotubes is more promising for medical applications, current toxicity studies focus primarily on environmental health concerns of pristine CNT. No comprehensive examination of functionalized CNT degradation-induced toxicity has been reported, but these are important questions as functionalized CNT have more promise as therapeutics and diagnostic tools.

Along with CNT aggregation as a function of improper macrophage uptake, the length of CNT has been implicated as a source of improper macrophage translocation. Poland et al. studied the effect of length on CNT toxicity by injecting MWCNT i.p. and observing carcinogenic mechanisms in the abdominal cavity and on the diaphragm [61]. The longer length (≥ 20 μm) CNT resulted in an inflammatory response within 24 h with consequent granuloma 7 days after injection. These longer lengths may cause carcinogenic effects such as mesotheliomas, if longer studies were conducted. In the same study, shorter lengths of i.p injected MWCNT were effectively taken up by macrophages with efficient phagocytosis. In terms of CNT for therapeutics, it can be deduced that SWCNT may be more favorable than MWCNT from a toxicology standpoint due to smaller size and length resulting in less aggregation and better uptake by macrophages. Hirano et al. further suggested that MWCNTs are recognized and interact with macrophage receptors on the plasma membrane and can rupture the membrane causing cytotoxicity and damage to the macrophage [72].

Methods of CNT fabrication and purification have been shown to increase toxic effects of CNT. Arc-discharge, laser ablation, chemical vapor deposition, and high-pressure carbon monoxide synthesis are methods commonly used to fabricate CNT [69,73]. These methods are often performed in the presence of metal catalysts with CNT growth occurring on solid supports. Impurities such as residual metal or supports and amorphous carbon in the final formulation have been thought to induce oxidative stress. On the other hand, extensive purification and treatment will also cause degradation of the nanotubes [73]. Therefore, a fine balance between synthetic methods and purification must be achieved to fabricate highly purified CNT for injection.

Low clearance and accumulation of CNT *in vivo* warrants studies to determine chronic exposure toxicity. Radiolabeled isotopes are commonly used to evaluate the *in vivo* performance of CNT [70,71]. However, due to isotopic decay and degradation of the label from the CNT, radiolabeling suffers from short study timeframes. Recently, Liu et al. and Cherukuri et al. have used Raman spectroscopy and near-infrared fluorescence, respectively, for intrinsic detection of CNT in tissue homogenates, urine, and feces samples [63,69]. Yang et al.

determined that pristine SWCNT remained stable up to 28 days in lungs, liver, and spleen by TEM visualization in mice that were injected i.v. with a high dose of CNT [64]. Therefore, estimation and evaluation of chronic exposure toxicity may be possible based on evaluation of CNT intrinsic properties.

In conclusion, toxicity concerns of general CNT use *in vivo* have stemmed from observed toxicity after delivery to the lungs. Current studies have shown that once in the bloodstream, intrinsic properties, propensity to aggregate, and slow clearance can lead to oxidative stress especially in the liver, lungs and spleen, ultimately resulting in inflammation. More importantly, the length of CNT has been shown to result in inefficient phagocytosis and damage to macrophages. Due to less aggregation and shorter lengths, SWCNT are better suited for *in vivo* applications over MWCNT. Functionalization of SWCNT further reduces aggregation; however, more thorough research on functionalized CNT toxicity will need to be conducted. Moreover, methodically conducted experiments that mimic chronic exposure to CNT will be more productive in the determination of CNT nanotoxicity.

2.3. Quantum dots

Quantum dots (QDs) are fluorescent semiconductor nanocrystals (~ 2-100 nm) with unique optical and electrical properties [74,75]. QDs possess high brightness due to their extinction coefficient and quantum yield, broad absorption characteristics, narrow line width in emission spectra, continuous and tunable emission maxima due to quantum size effects, and a longer fluorescence lifetime with negligible photobleaching over minutes to hours [76-79]. These properties make QDs advantageous in biotechnology and medical applications as optimal fluorophores for *in vivo* biomedical imaging and for targeting specific cells (e.g., labeling neoplastic cells, peroxisomes, DNA, and cell membrane receptors) after conjugation with specific bioactive moieties [80-85]. These applications are predicted to grow because of their numerous advantages over alternative biological labeling moieties (e.g. fluorescent dyes and radioisotopes).

QDs have a metalloid crystalline core (e.g. CdSe) and a shell (e.g. ZnS) that shields the core (Fig. 2) [86]. The core consists of a variety of metal complexes such as semiconductors, noble metals, and magnetic transition metals [87,88]. The shell, formed on the metalloid core during synthesis in organic solvents, makes QDs hydrophobic, and hence limits their use in biological applications. To make QDs biocompatible, secondary coatings are added that improve water solubility, QD core durability, and suspension characteristics. These organic coatings can be used towards targeting biological components for diagnostic or therapeutic purposes [89].

The safety of metals and their use *in vivo* is an ongoing topic of debate. Divalent cations are known to be toxic, even at low concentrations in the body. Due to reabsorption and accumulation in the kidneys, the main problem with heavy metals is nephrotoxicity [90]. For example, gadolinium-based contrast agents for clinical MRI analysis have been reported to be associated with acute renal failure [91]. Some researchers have considered QDs to be inert structures where the metallic core is safely passivated, while others have dismissed the use of QDs *in vivo* because of potential toxicity. Cadmium and selenium, two of the most used metals in QD cores, are known to cause acute and chronic toxicities in vertebrates and are of significant health and environmental concern [92]. Cadmium has a biological half-life of 15-20 years in humans, accumulates in tissues, and can cross the blood-brain barrier and the placenta. In the case of QDs, release of Cd^{2+} from the core and generation of ROS and oxidative stress appears to be the mechanism of *in vivo* toxicity [93,94]. Although researchers agree on the toxicity of Cd^{2+}, conflicts remain on the use of QDs *in vivo*. Studies have suggested that the shell (most commonly ZnS) along with secondary coatings prevent Cd^{2+} leakage from the core [95]. Others suggest that Cd^{2+} can leak from the core with QD complex instability. Arguments of QD safety can be somewhat confusing because of the variety of QDs being synthesized and considered.

Studies concluding that QDs are toxic indicate that toxicity may originate from QD stability, size, surface charge, and type of surface coating.

Possibly the most important aspect of QD toxicity is the stability of the core/shell/coating complex. Stable complexes should protect the body from core metal toxicity. Several studies suggested that under oxidative and photolytic conditions, QD are labile and subject to degradation, thus exposing the potentially toxic shell material, intact core metalloid complex, and core metal components (e.g. Cd, Se) [75]. It has been reported that Cd^{2+} released under oxidative attack can bind to the sulfhydryl groups of critical mitochondria proteins leading to mitochondria dysfunction and ultimately cell poisoning [92]. Addition of the secondary surface coating renders QDs biocompatible and can help protect against degradation of QDs, but the method of functionalization (e.g. electrostatic, adsorption, multivalent chelation, or covalent bonding) and the coating composition are important when considering QD durability, stability, and *in vivo* reactivity [75]. Interestingly, a recent communication by Mancinci et al. suggests an oxidative mechanism wherein hypochlorous acid and hydrogen peroxide, both produced by phagocytic cells, may diffuse across polymeric secondary coatings and cause QD lattice defects and solubility of the core [96]. Therefore, there may be *in vivo* oxidative mechanisms of QD degradation regardless of the presence of stable secondary coatings.

Some studies have suggested that QD size is the source of toxicity regardless of the presence or absence of secondary coating. Shiohara et al. studied the cytotoxicity of CdSe/ZnS coated with mercaptoundecanoic acid (MUA), resulting in a carboxyl-QD, in three different cell lines. They determined that increased cell death correlated with a decrease in QD size even at low concentrations [78]. These results were consistent with studies by Zhang et al. supporting increased cytotoxicity with small sized QDs in rat hepatocytes [97].

Since a secondary coating is almost always used for *in vivo* applications, many studies have focused on what roles coatings play on QD stability relative to nanoparticle toxicity [95]. To assess the importance of surface charge, many groups have studied the toxicity of cationic (amine) and anionic (carboxyl) secondary coatings on QDs. Geys et al. conducted an acute *in vivo* toxicity study on amine- and carboxyl-coated commercially available CdSe/ZnS QDs. Low doses of 1.44 pmol or 14.4 pmol of both types did not elicit acute adverse effects. However, at high doses the QDs caused pulmonary vascular thrombosis, with carboxyl-QDs being more potent in inducing this effect than amine-QDs. Because fibrin fibers were present in the thrombi and because pretreatment with heparin abolished the thrombotic effects, they speculated that negatively charged QDs activated the coagulation cascade via contact activation [98]. Hoshino et al. coated CdSe/ZnS with mercaptoundecanoic acid (MUA) (QD-COOH), cysteamine (QD-NH_2), thioglycerol (QD-OH), QD-OH/COOH and QD-NH_2/OH to assess the effects of surface charge on the cytotoxicity of hepatocytes. They found that the highly negative charge QD-COOH induced DNA damage after 2 h while the other types have no significant cellular damage [99]. They suggest that after endocytosis, the acidic nature of the endosome creates QD-COOH instability in the cell where QDs can easily aggregate. However, only amine-QDs were stable in acidic conditions (100 nM QD incubated in glycine buffer (pH = 3.0) for 30 min at room temperature) [100]. Overall, a balance of surface charge should be considered in evaluating potential stability and toxicity issues.

The type of secondary surface coating may affect the toxicity of the QD complex. Polyethylene glycol (PEG), a common pharmaceutical excipient, is used extensively in commercially available QDs. Ballou et al. prepared QDs coated with amphiphilic polyacrylic acid and simultaneously conjugated to different molecular weights of PEG (750 and 5000). By monitoring QDs in mice with fluorescent imaging, they found that significant liver uptake was visible even at 1 min using (750)-PEG-QD, but completely cleared away after 1 h, while (5000)-PEG-QD was absorbed by liver in 1-3 h post-injection. They determined no significant liver

toxicity; however, they found differences in accumulation and clearance [101]. A longer study (28 days) was carried out by Yang et al. on a commercially available QD, (5000)-PEG-ZnS/CdTe (QD705, about 13 nm). After a single i.v. injection in mice, they demonstrated that the liver and spleen were the main accumulative organs. QDs were not detected in either urine or feces, which would suggest accumulation of QDs with high molecular weight PEG [89]. When PEG-QDs are injected subcutaneously, QDs clear from the site of injection and accumulate in the regional lymph nodes along with the liver, spleen, and kidney [102]. Therefore, the type of injection would need to be considered for QD accumulation and toxicity [75,103]. Albumin is another commonly used secondary coating for QD *in vivo* applications. Fisher et al. coated CdSe/ZnS with MUA/ lysine to form QD-LM (about 25 nm) or coated the QD-LM with bovine serum albumin (BSA) to form QD-LM-BSA (about 80 nm). After i.v. injection in the rat, both QDs were mainly detected in the liver, although QDs were detected in the spleen, lung, kidney, and bone marrow. However, the QD-LMBSA liver uptake was greater (99%) relative to the QD-LM uptake (40%). This corresponded with a faster blood clearance of QD-LM-BSA. Neither type of QD was detected in the urine or feces up to 10 days, which would suggest accumulation in the liver, especially with BSA conjugation [104]. Overall, most studies have indicated that differences in coating material and size lead to changes in pharmacokinetics and can potentially cause toxicity.

In conclusion, the potential toxic effects of QDs have become a hot issue that must be further addressed before clinical applications would be possible. Most studies recommend that not all QDs are similar in their toxicity, and toxicity of differing QDs must be considered individually. The adverse effects of QD can be mitigated or eliminated by proper choices of coating materials and modification techniques that reduce QD instability.

2.4. Gold nanoparticles

Due to a straightforward synthesis, stability, and ease of incorporation of functional groups for targeting capabilities, gold nanomaterials have great application in gene and protein delivery, biological imaging, cancer treatments, and in implants (e.g. pacemakers and stents) [105-107]. Additionally, gold has been used as anti-inflammatory and anti-rheumatic agents (Auranofin® and Tauredon®) in the treatment of rheumatoid arthritis [107]. Many studies have suggested that gold nanomaterials are bioinert and can be used safely. This thought may be due to the established safety of bulk gold materials, but as the size decreases into the nanoscopic dimensions, gold will behave very differently than in bulk. Some research has found gold to be toxic in the body, where elemental gold can undergo oxidation or become soluble by cyanidation [108,109]. Studies have shown that gold is heavily taken up by the kidneys, causing nephrotoxicity, and can also initiate eryptosis (erythrocyte suicidal death) [110,111]. Studies have suggested that the size, surface charge, and shape are key factors related to potential toxicity of medicinal gold complexes.

Several studies have examined the effect of gold nanoparticle size on toxicity. Specifically, gold nanoclusters (illustrated in Fig. 2) of 1.4 nm have been shown to selectively and irreversibly bind to the major grooves of B-DNA and cause increased cytotoxicity compared to larger particles (18 nm). The lack of interaction of larger particles with DNA is suggested to be due to steric hindrance. While gold nanoclusters may be very effective cancer treatments, healthy cells would also be affected potentially causing toxicity [112,113]. Similarly, Pan et al. studied the size-dependent cytotoxicity of gold nanoparticles (water soluble and stabilized with triphenylphosphine derivatives) on several different cell lines. They discovered that nanoclusters of 1.4 nm exhibited increased cytotoxicity (IC_{50}= 30 and 46 μM), whereas nanoclusters of 0.8, 1.2, and 1.8 nm where four to six-fold less toxic. Larger sizes (15 nm) exhibited no cytotoxicity even at high concentrations (6.3 mM) [114]. Moreover, Chithrani et al. examined the uptake of 14, 50, and 74 nm sized citric acid ligand stabilized gold

nanoparticles into HeLa cells and determined that 50-nm spheres were more quickly taken up by endocytosis than both the smaller and larger sizes [115]. They further studied the rate of exocytosis of transferrin-coated gold nanoparticles and determined that the rate of exocytosis was size-dependent with more accumulation of larger gold nanoparticles in the cell [116]. When studying the effect of gold nanoparticle size after i.v. injection of colloidal gold in mice, smaller particles (10-50 nm) were found to disperse quickly to almost all tissues, mainly accumulating in the liver, lungs, spleen, and kidneys at 24 h post injection. Larger particles (100-200 nm) were found in the liver, lungs, spleen, and kidneys, but they were not as widely dispersed into other tissues as were the smaller particles. These studies concluded a size-dependent distribution and potential toxicity of smaller (< 50 nm) gold nanoparticles [105, 117].

To condense and deliver pDNA successfully, synthetic gene transfection vectors generally must be cationic. Sandhu et al. determined that cationic gold nanoparticles were 8-fold more efficient as a non-viral gene vector over the commonly used polymer polyethyleneimine [118]. Goodman et al. examined the effects of gold nanoparticle surface charge on cytotoxicity by studying cationic (amine) and anionic (carboxyl) gold nanoparticles on Cos-1 cells, red blood cells, and *E. coli*. It was concluded that cationic gold particles were moderately toxic and anionic particles were nontoxic, suggesting the initial electrostatic binding of the particles to the negatively charged cell membrane as a probable mechanism of toxicity and that electrostatic repulsion may limit anionic and neutral particle interaction with the cell surface [119]. So, cationic gold nanoparticles can be expected to exhibit more toxic effects relative to anionic particles. However, this would need to be performed *in vivo* for a better assessment of the effects of surface charge on biological interaction and gold nanoparticle toxicity.

Gold nanomaterials can be found in many different shapes, especially as spherical clusters and nanorods. Along with size, Chithrani et al. studied the effect of shape on the cellular uptake of gold nanoparticles. They concluded that both nanorods (74 × 14 nm) and spherical particles (74 and 14 nm) are taken up by cells; however, nanorod uptake is slower relative to spherical particles in HeLa cells [115]. Wang et al. further concluded that nanorods are more cytotoxic than spherical gold nanomaterials to human HeCaT keratinocytes [120]. Although both studies concluded differences between spherical particles and nanorods, both Chithrani et al. and Wang et al. suggested that the more important difference in cellular uptake rates and cytotoxicity was the use of different chemistries to stabilize the gold nanomaterials. During synthesis, cetyltrimethylammonium bromide (CTAB) is a commonly used cationic surfactant to stabilize gold nanorods. Although aggregation of gold nanorods has been shown intercellularly, nanospheres also show aggregation without causing cytotoxicity [120,121]. This led to future investigation into the toxicity of CTAB with several studies recognizing it as a main source of toxicity of gold nanorods [120-122]. CTAB surface reduction has been achieved by displacement of CTAB with PEG, citric acid ligands, and by coating gold nanoparticle surfaces with transferrin. Studies have concluded that these modified gold nanoparticles do not exhibit cytotoxic effects, although the amount of displaced CTAB is difficult to quantify with current analytical methods [115,120-123]. *In vivo* studies on the effects of CTAB and aggregation of gold nanomaterials will need to be evaluated, as well as more complete studies on the effects of gold nanoparticle shape on toxicity. Additionally, alternative means to stabilize gold nanorods need to be considered.

In conclusion, gold nanoparticles have numerous medical applications and traditionally have been thought of as bioinert since bulk gold has been deemed as such. However, more studies are suggesting potential toxicity concerns for gold nanoparticles due to their size, surface charge, and shape - the same unique properties that make them so appealing for medical applications. Unfortunately, most of the studies suggesting toxicity of gold nanoparticles come

from *in vitro* experimentation. Thorough distribution and toxicity studies are critically needed to fully evaluate gold nanoparticles for *in vivo* use.

3. Analysis of nanomaterial toxicity

As current studies show conflicting results on safety and biocompatibility of nanomaterials, it is recognized that validation of analytical methods used to determine toxicity is necessary. Since engineered nanomaterials are so unique and complex, standardizing analytical methods for nanoparticle characterization is rather complicated. As reviewed by Hall et al., complete evaluation of nanoparticles for therapeutic use include thorough physicochemical property characterization, sterility and pyrogenicity assessment, biodistribution, and toxicity determination (both *in vitro* and *in vivo*) [124].

Powers et al. recognized that material properties are complicated by the behavior of the material in biological environments and that traditional methods of characterizing powder forms of nanomaterials may be ineffective for characterizing materials in biological systems. They proposed key characteristics that should be evaluated and reviewed analytical methods of measuring nanomaterial size and shape, dispersion, physicochemical properties, surface area, porosity, and surface [14]. In particular, it was suggested that transmission electron microscopy (TEM) and dynamic light scattering (DLS) would be suitable techniques for size and shape measurements in biological systems [14,124]. TEM and DLS was successfully applied to a study by Murdock et al. to characterize aggregation of various nanomaterials in water and culture media (with and without serum) [125].

In vivo distribution and toxicity examinations should include studies such as initial disposition, therapeutic efficacy, and dose-response studies, at a minimum. Distribution studies have been conducted where nanomaterials were tracked in the blood and tissues by conjugating the material with a radiolabel or organic dye, if direct detection of the material was not achievable. Degradation of the conjugate and the inevitable radiolabel decay or dye quenching limit this technique for toxicity studies where long-term evaluations are necessary. If possible, detection based on the material intrinsic properties is preferred, especially if the material is stable, to avoid misleading results [69]. However, since metabolism and degradation is often observed with nanomaterials used *in vivo*, detection strategies for analysis of all major parts of the nanomaterial, although challenging, would be the most ideal approach (i.e. a multi-indicator approach) [6,124]. Currently, toxicity assessments are performed by probing for inflammation. Tissue histological examination for immune response is traditionally performed, with some studies of overall animal behavior and weight loss as indicators of material intolerance. Moreover, analysis of biomarkers of inflammation, cell viability, and oxidative stress generation would enhance mechanistic studies into nanomaterial toxicity [65]. A summary of the conducted toxicity *in vivo* toxicity studies of the four presented nanomaterials is displayed in Table 2.

4. Summary, conclusions and future challenges

Although studies are conflicting regarding the magnitude and mechanisms of nanomaterial toxicity, it is evident that some nanomaterials that were previously considered biocompatible due to safety of the bulk material may indeed be toxic. Nanomaterial size, shape, surface chemistry, and degree of aggregation influence the production of free radicals and subsequent oxidative stress. Nanoparticle toxicology is a relatively young field, and the bulk of reports have focused on acute toxicity. Long-term toxicity of the materials and examination of chronic exposure are critical to understanding the toxicology of nanomaterials *in vivo*. Evaluation of toxicity has proven to be challenging as several factors may be working in tandem to cause nanoparticle toxicity. Furthermore, as nanomaterials are inherently quite complex, many unexpected interactions (based on bulk properties) with biological components may arise.

However, with appropriately validated analytical methods and carefully designed experimentation, the mechanisms of toxicity may become clearer so that nanomaterials can safely be used as therapeutics and as diagnostic tools.

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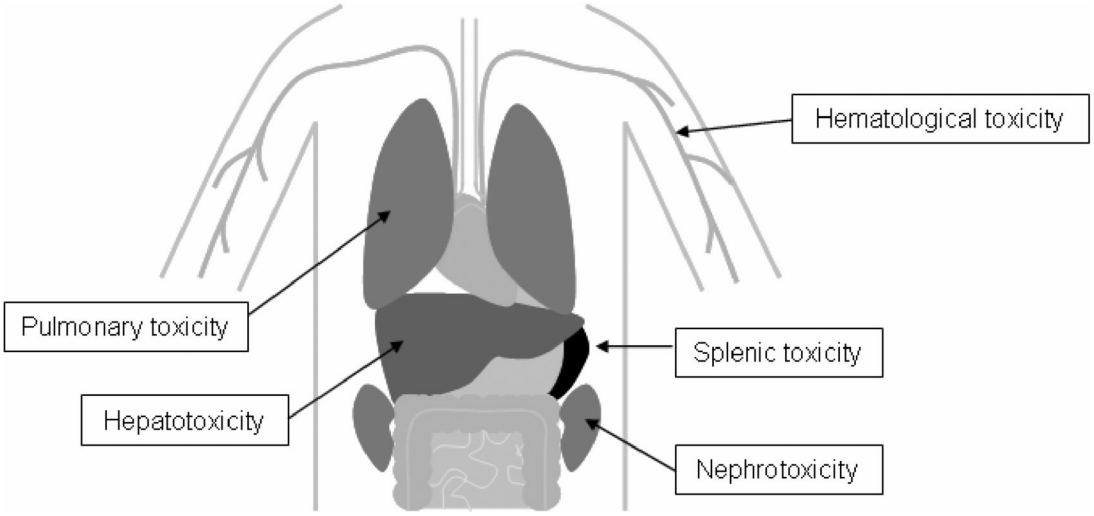

Figure 1.
Major forms of *in vivo* nanomaterial toxicity

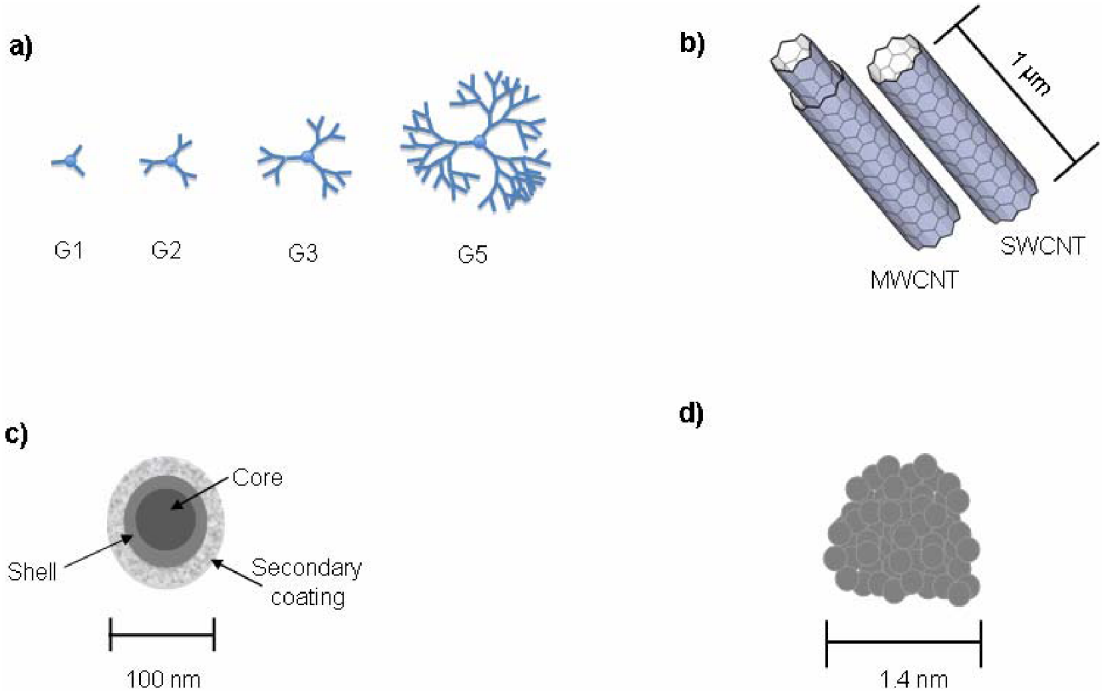

Figure 2.
Schematic of the structures of a) dendrimers of varying generations, b) carbon nanotubes, c) a quantum dot and d) a gold nanocluster

Table 1

Summary of primary causes of nanomaterial toxicity *in vivo*

Nanomaterial Type	Primary Toxicity Concerns	Reference
Dendrimers	Surface charge	[24] [25] [36] [37]
	Size (generation)	[24]
Carbon nanotubes	Size (aggregation)	[63-68]
	Length	[61] [62] [72]
Quantum dots	Stability of core/shell/coating	[75] [92] [96]
	Type and surface charge of coating	[75] [89] [99-101] [103] [104]
Gold nanoparticles	Size	[105] [112-117]
	Shape	[115] [120-122]

Table 2

Summary of the conducted *in vivo* toxicity studies

Nanomaterial	Toxicity Study	Analyzed Parameters	Study length	Reference
Dendrimers	General toxicity	Behavior, weight change, mortality	24 h - 6 months	[24] [37] [40] [45]
	Hematological toxicity	Complete blood count	7 days	[25]
	Immunogenicity	IP, ODD, Antibody (IgG) titre by ELISA	21-52 days	[24] [25]
	Hepatotoxicity	Histology, serum ALT	48 h - 6 months	[24] [41]
	Nephrotoxicity	BUN	48 h - 6 weeks	[41]
Carbon nanotubes	PK and distribution	Radiolabel	24 h -18 days	[70] [71]
		TEM of tissues, urine & feces	90 days	[64] [65] [61]
		Raman spectroscopy	90 days	[64] [69]
		Near-infrared fluorescence	24 h	[63]
	Tissue inflammation	Histology	7 - 90 days	[61] [64] [68]
	Hepatic function	Serum LDH, TBIL, ALT, AST	90 days	[64]
	Oxidative stress	Tissue glutathione & MDA levels	90 days	[64]
Quantum dots	PK and distribution	TEM of tissues	90 min - 24 h	[101] [104]
		Weight change	28 days	[89]
		ICP-MS of tissue homogenates	3 hrs - 28 days	[89] [104]
		Fluorescence spectroscopy	28 days	[89]
Gold nanoparticles	PK and distribution	ICP-MS of tissue homogenates	24 h	[105] [117]

*IP = Immunoprecipitation; ODD = Ouchterlony double diffusion; ALT = alanine aminotransferase; BUN = blood urea nitrogen; LDH = lactose dehydrogenase; TBIL = total bilirubin; AST = aspartate aminotransferase; MDA = malondialdehyde