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Original Research

Immune Responses of Mammals to Foreign Nanomaterials, Oxidative Stress and Inflammation Assays, Toxicity of Nanomaterials to Living Cells, and Nanomedicinal Applications

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Abstract:

As a consequence of their increase in annual production and widespread distribution in the environment, nanoparticles potentially pose a significant public health risk. The sought-after catalytic activity granted by their physiochemical properties doubles as a hazard to physiological processes following exposure through inhalation, oral, transdermal, subcutaneous, and intravenous uptake. Upon uptake into the body, their size, morphology, surface charge, coating, and chemical composition augment the response of biological systems to the materials and enhance their toxicity. Identification of each property is necessary to predict the harm imposed by foreign nanomaterials in the body. Assay methods ranging from endotoxin and lactate dehydrogenase (LDH) signaling to apoptosis and oxidative stress detection supply valuable techniques for exposing biomarkers of nanoparticle-induced cellular damage. Spectroscopic investigation of epithelial barrier permeation and distribution within living cells reveals the proclivity of nanoparticles to penetrate the body's natural defensive boundaries and deposit themselves in cytotoxic locations. Combination of the various characterization methodologies and assays is required for every new nanoparticulate system despite preexisting data for similar systems due to the lack of deterministic trends among investigated nanoparticles. The propensity of nanomaterials to denature proteins and oxidize substrates in their local environment generates significant concern for the applicability of several traditional in vitro assays, and the modification of susceptible approaches into novel methods suitable for the evaluation of nanoparticles comprises the focus of future work centered on nanoparticle toxicity analysis.

Keywords: Nanomaterials, Toxicity, Living Cells, Immune Responses, Oxidative Stress, Inflammation Assays.

Introduction:

Owing to their unique optical, magnetic, and thermal properties, nanomaterials (NMs) have been widely explored in radiation diagnosis and therapy. For instance, carbon nanotubes (CNTs) are used for Raman and photoacoustic imaging and thermal therapy; magnetic nanoparticles (MNPs) for magnetic resonance imaging (MRI) and thermal therapy; and gold nanoparticles (GNPs) for x-ray computed tomography (CT) contrast agents. However, with recent nanomedicine applications, there is a growing concern about the toxic effects generated by NMs. Because these theranostic nanoparticles will be inevitably injected into the human body when they are used in clinics, the related toxicity issues must be addressed before any human use. The toxicity of NMs in living cells and mammals has been frequently reported [1-3]; the results of preliminary studies reveal that multiple biological mechanisms may be involved. However, our knowledge about the toxicologic mechanism of NMs and the correlation between their physicochemical properties and toxicity is very limited.

NMs have small size (1–100 nm) and extremely large surface areas; thus, large proportions of their atoms or molecules are exposed on the surface (Oberdörster et al. 2005). This renders them with strong surface energy and makes them be prone to react with surrounding molecules, including biomolecules. Therefore, unlike for bulk materials or small-molecule toxicants, the dimensions of NMs are an important variant determining their toxicity. NMs can enter cells by energy-dependent endocytosis or phagocytosis and by direct cell membrane penetration [4, 5]. NMs are also prone to aggregate and accumulate in various organs, resulting in a low elimination rate. They can also translocate to other regions from the portal of entry and cause a wide range of damages. Because of their dimensions, they may escape the innate immune system and macrophages. All these traits have raised more concerns about NMs' potential toxicities. The shape and surface chemistry of NMs are crucial factors that determine their interactions with biological molecules, capability to penetrate cell membranes, and in vivo distributions. Therefore, these properties control their potential toxicities. Examples include gold NMs with various shapes (Wang et al. 2008) and CNTs with various surface chemistries.

Toxicity of Nanomaterials to Living cells:

Cell Uptake and cellular translocation:

NMs can enter living cells through endocytosis (Mu et al. 2010; Sahay et al. 2010). During the process, NMs are engulfed into endosomes formed by cell membrane invaginations and then redistributed into various cell organelles. Phagocytosis is one of the endocytic pathways for NMs' cellular uptake. This process occurs in phagocytes, such as macrophages and monocytes, and to a much lower extent in fibroblasts, epithelial cells, and endothelial cells. The uptake of particles by phagocytes does not depend on the size of the particles. Yet, the particle's shape at the attaching point is a crucial factor for macrophage uptake [6-8]. NMs usually enter cells through more than one pathway, including clathrin- and caveolae-dependent endocytosis, clathrin- and caveolae-independent endocytosis, and macropinocytosis. Size, shape, charge, and functional groups collectively determine the cellular uptake of NMs. The uptake of GNPs by HeLa cells heavily depends on the particles' size, and the maximum amount of uptake was found for GNPs with a diameter of 50 nm. Particles between 14 and 100 nm were trapped in vesicles inside the cell and did not enter the nucleus.

Furthermore, NMs with higher aspect ratios enter cells faster compared with their more symmetrical, cylindrical particle counterparts. Additionally, sphere-shaped nanostructures enter cells compared with rod-shaped ones, showing the effects of curvature of NMs. Surface charge also plays an important role in the uptake of NMs. Nanorods coated with a negatively charged layer are not taken up by HeLa cells as quickly as are nanorods with a positively charged layer [9]. This outcome may be related to electrostatic interactions with the negatively charged cell surface. Because the functional groups on the

surface of NMs may change the particles' interactions with cells, chemically modified NMs might have different cellular uptake rates than unmodified ones do.

Additionally, having anionic surfactants and polyethylene glycol on the surface may prevent the cellular uptake of nanorods. Serum protein adsorption may also influence cellular uptake. One study shows that serum proteins adsorbed on the surface of NMs may enhance the uptake. However, another study shows that cellular uptake of carbon NMs is much higher in serum-free culture medium than in culture medium with serum [10]. Most NMs are too big to enter the nucleus. For example, GNPs that enter the human dermal fibroblasts gather in the lysosome, and gold nanorods become trapped in vesicles in cells (Hauck et al. 2008). However, peptide-BSA GNPs can enter the nuclei of HeLa cells (Franzen et al. 2004). Single- and multi-walled CNTs (SWCNTs and MWCNTs) can also enter cell nuclei .

Oxidative Stress Perturbation by Nanomaterials:

To counteract the effects of reactive oxygen species (ROS), organisms have several distinct antioxidants, including superoxide dismutase, catalase, ascorbic acid, and glutathione. But when the antioxidant defense is overwhelmed, abnormal oxidative stress will be the consequence. The oxidative stress–induced damages include cellular membrane injury, DNA damage, protein denaturation, mitochondrial perturbation, cell apoptosis, and necrosis [11]. Perturbation of the oxidative stress balance has been proposed to be a general mechanism for nanotoxicity. Several types of fullerenes (C60) generate superoxide anions in water, possibly causing oxidative damage to cell membranes and subsequent cell death (Sayes et al. 2004). Additionally, pristine SWCNTs cause oxidative stress to cells and the inflammatory response. Silver nanoparticles (SNPs) also cause cell damage by increasing ROS production and reducing ATP generation, leading to DNA damage.

Cellular Perturbations of Nanomaterials:

A 4-day incubation of GNPs and dermal fibroblasts significantly reduces the cells' proliferation, partly because of the GNPs' effects on actin fibrils (Pernodet et al. 2006). Additionally, a maximal dose or chronic low dose of C60(OH)24 inhibits the growth of human umbilical vein endothelial cells, causing autophagic cell death (Iwai and Yamawaki 2006). Furthermore, three different types of CNTs (SWCNTs, 50% SWCNTs + 30% MWCNTs + 20% C60, MWCNTs) strongly affect the proliferation of U937 monocytic cells, with little effect on the cells' viability [12]. COOH-functionalized SWCNTs and MWCNTs also significantly affect mesenchymal stem cells' (MSCs) proliferation, which might be related to the binding of CNTs to nutrients in the culture medium (Mooney et al. 2008) and their effects on the bone morphogenetic protein (BMP) signaling pathway. Once GNPs enter human dermal blasts, actin fibers disappear. Carboxylated MWCNTs and SWCNTs inhibit the osteogenic differentiation of MS alkaline phosphatase (ALP) activity. During adipocyte differentiation of MSCs, ALP activity is also strongly inhibited on day 14. Quantitative polymerase chain reaction analysis shows that several differentiation genes are down-regulated after treatment with CNTs. Studies on the effects of several CNTs (i.e., SWCNTs, double-walled CNTs, MWCNTs) on osteo-blasts revealed that CNTs inhibit mineralized nodule formation during the final stage of cell differentiation.

Effects on cellular Signaling:

NMs have been repeatedly reported to activate or affect cellular signaling pathways, although their direct cellular targets are unknown. Nuclear factor-kappa B (NF-κB). Several NMs can generate oxi- dative stress in cells, thus influencing signaling pathways, such as those of MAP kinases and NF-κB. When human keratinocytes are treated with SWCNTs, NF-κB is activated in a dose-dependent manner, which might relate to the activation of stress-related kinases [13]. MWCNTs have also been reported to

induce cell ROS and IL-8, along with the activation of NF-κB, which may lead to the death of A549 cells. MAPK. CNTs can activate MAPK/ERK signal transduction, thus promoting neurite outgrowths in DRG neurons and PC12h cells. Similar results are obtained when PC12 cells are treated with iron oxide NMs. TiO2 NMs induced phosphorylation of p38 MAPK and Erk-1/2 and inhibited human polymorphonuclear neutrophils apoptosis. MNPs coated with specific ligands activate the MAPK signaling pathway when a magnetic field is applied. Furthermore, GNPs modulate osteogenic and adipocytic differentiation of MSCs through the p38 MAPK signaling pathway.

Immune Responses of Mammals to Foreign Nanomaterials:

The immunotoxicity of Nanomaterials should be well evaluated before biomedical applications in humans. Inhaled MWCNTs can suppress systemic immune function via activation of cyclooxygenase enzymes in the spleen, which results from the activation of TGF-β in the lungs [14]. Bound proteins on NMs result in different immune responses. Proteins around the particle determine the NM uptake by various cells of the immune system and influence how they interact with blood components. If NMs' surfaces are not modified to prevent the adsorption of opsonins, then a rapid removal by macrophage cells will occur. Other immune cells and removal mechanisms would be simultaneously stimulated to remove NMs from the bloodstream.

Physicochemical properties of NMs influence their immunotoxicity. Smaller Nanomaterials (~25 nm) travel through the lymphatic system more readily than do larger particles (~100 nm), and they accumulate in the lymph nodes' resident dendritic cells. The surface charge of liposomes also makes a difference. Cationic liposomes generate a greater immune response than do anionic or neutral liposomes. Solid lipid nanoparticle (SLN)–encapsulated antisense oligode-oxyribonucleotide G3139 had greater immunostimulatory and antitumor activity than did free (i.e., nonencapsulated) G3139. Because of SLNs' small size, tumor resident macrophages and dendritic cells take them up efficiently.

Organ Damage induced by nM Administration:

Nanomaterials can penetrate various barriers and enter different organs. The oxidative stress signaling activated by Nanomaterials will directly affect the functions of organs in which they are located and may cause systematic toxicity through blood circulation of these stress signaling molecules MNPs. The toxicity of MNPs can be decreased by modification. For example, the toxicity of MNPs was effectively lowered by encapsulating them in poly(d,l-lactide) (Gajdosíková et al. 2006). Many MNPs have shown potential as tumor targeting and imaging molecules without much toxicity (Muldoon et al. 2005; Weissleder et al. 1989); however, some of them cause acute toxicity [14].

GNPs. GNPs ranging from 8 to 37 nm induce severe sickness in mice. Pathologic examination of the major organs of the mice revealed an increase of Kupffer cells in the liver, a loss of structural integrity in the lungs, and diffusion of white pulp in the spleen. The pathologic abnormality was associated with the presence of GNPs at the diseased sites. After modifying the surface of the GNPs by incorporating immunogenic peptides, the toxicity of the GNPs was reduced. The toxicity of 13.5 nm GNPs in mice was evaluated by using different administration models and various doses. At low concentrations, GNPs caused no toxicity in vivo. At high concentrations, they caused decreases in body weight, red blood cell numbers, and haematocrit values. The mice that received oral and intra-peritoneal doses experienced higher toxicity than those dosed through tail vein injections [15].

CNTs. CNTs can reach the pleural cavity or the peritoneum, resulting in chronic granulomatous inflammation, which might be the forerunner of mesothelioma. When mice inhaled CNTs, dose-dependent epithelioid granulomas were found (Lam et al. 2004). Furthermore, the inhaled CNTs induce secondary platelet activation in the

systemic circulation and promote atherosclerosis [15]. CNTs could also induce reversible reproductive toxicity in adult males.

Methods for Assessing toxicity of NMs:

The methods used to evaluate the toxicity of NMs are different from those used for small molecule toxicants because of NMs' unique physicochemical properties. Methods have been developed to study the in vitro and in vivo toxicity of NMs. For instance, the imaging approaches in medical use are also applied in toxicity studies, and the inorganic nature of some NMs enables various elemental analysis methods to be used. However, more efficient methods still need to be developed for fast and thorough evaluation of nanotoxicity in biological matrices.

Evaluating the In Vitro toxicity of NMs:

Cell Uptake:

Transmission electron microscopy (TEM) is a method used for qualitative analysis. Detailed ultrastructural information, such as the size and morphology of NMs and the location of NMs inside cells, can be obtained. TEM also provides information on cell uptake pathways. However, its application is limited to electron-dense NMs.

Inductively coupled plasma atomic emission spectroscopy (ICP-AES) is a method used to quantify internalized NMs according to each element's emission spectrum. It can be used with high sensitivity to identify the elements in NMs [16]. However, this technique cannot provide any spatial information. Inductively coupled plasma mass spectrometry (ICP-MS) is highly sensitive and capable of detecting the presence of a range of metals and several nonmetals at ppt concentrations. Similar to ICP-AES, sample preparation before NM component quantification is required. Compared with several other quantitative analytical methods, ICP-MS has a lower detection limit, wider dynamic range, and higher precision. This method has the advantage of enabling the simultaneous determination of multiple elements. Fluorescence detection can be used to quantify NM uptake and observe NMs' location. Quantitative assessment can be achieved in a manner similar to that used for ICP-AES by using bulk fluorescence or, on a single cell, confocal fluorescence. It is also possible to correlate NMs counting directly with cell numbers or sort cells on the basis of NM uptake by using fluorescence-activated cell sorting (FACS). Detection is limited by the fluorescent properties of the NM species and the collection efficiency of the instrument. The spatial resolution is diffraction-limited, 200 nm at best [17]. The inherent fluorescent properties of certain NMs allow simple analysis such as that of the uptake of QDs into human MSCs.

Cell Proliferation and Apoptosis:

Cellular reduction of tetrazolium salts to produce formazan dyes is widely used as an in vitro nanotoxicity assessment. The production of formazan-based dyes is monitored by optical absorbance as a measurement of cellular metabolism, which is used to assess the percentage of metabolically active cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is one of the most commonly used dyes for this purpose. The MTT method, however, suffers from one disadvantage—that formazan is insoluble but should be dissolved before the absorbance measurement—which seems to limit its application. Similar to MTT, XTT, MTS, and WST-1 are substrates of mitochondrial dehydrogenase and produce a highly water-soluble formazan from metabolically active cells, allowing a direct and user-friendly colorimetric measurement of cell viability and proliferation. The alamar blue assay has been used in colorimetric metabolic assay used to ascertain cell proliferation [18] via the bioreduction of the nonfluorescent alamar blue dye to a pink fluorescent dye. The active cell number is counted by either optical absorbance measurements or fluorescence detection.

Cell apoptosis assays include the annexin-V assay, DNA laddering, Comet assay, and TUNEL assay. Annexin-V is a phosphatidylserine (PS)-specific binding substrate that translocates to the exterior of cells in the early stage of apoptosis because of the restructuring of the plasma membrane. When

labeled with a fluorescent tag, such as FITC, annexin-V can be used as a probe to detect PS exposed in combination with propidium iodide. The apoptotic cell amount can then be measured by flow cytometry. Inspection of morphologic changes during apoptosis is the least instrumentally intensive method for characterizing apoptosis, requiring only a light microscope and visual inspection. Despite the low cost, this method is not used widely in nanotoxicology because of its time-intensive nature.

Mechanisms of Toxicity:

Nanoparticle toxicity stems primarily from the particle's physiochemical properties and individual architecture. Size, shape, surface charge, coating, and chemical constituency all play key roles in determining the particles' uptake and biocompatibility. Established by the National Nanotechnology Initiative, nanoparticles are defined as having characteristic dimensions below 100 nm, but their size is generally recognized to include particles with dimensions of up to 1 μm [19]. In the lower strata of the size regime, particularly below 100 nm, nanoparticles can take advantage of the enhanced permeation and retention (EPR) effect attributed to highly vascularized tissues such as tumors which makes the size distribution desirable among the pharmacological community. Additionally, as the size of nanoparticles decline, the energy barrier associated with uptake is also reduced, allowing for enhanced transdermal migration and cellular penetration. Decreasing the total surface area exposed to a phagocyte lowers the energy penalty required for energy-dependent endocytosis and raises the probability of phagocytic consumption. The relative increase in particle surface area linked to a reduction in volume also heightens the particle's reactivity and enables further uptake through receptor-mediated endocytosis and nonphagocytic mechanisms. The shape of nanoparticles plays a significant role in the progression of a material through the body in as much as the particles' size. Morphologies can vary from homogeneous and heterogeneous solid spheres to hollow micellular rods depending on the material in question and the synthesis pathway employed [20]. Spherical nanoparticles are reported to undergo phagocytosis and excretion at faster rates than their high aspect ratio counterparts. The phenomenon is assumed to be due to the minimization of contact area intersecting the cell membrane and the resulting added energy penalty associated with internalization. When the long axis of a cylindrical particle is aligned perpendicular to the membrane surface, the enlarged and unsymmetrical deformation needed to envelope the incident particle is assumed to be energetically unfavorable in comparison to uniform spheres. In another conformation, if the face of the tube first imposes on the cell membrane, phagocytosis may commence without the opportunity for completion. The event can lead to frustrated phagocytosis with eventual cell rupture and localized inflammation as in the case of asbestos. In a countervailing study, however, Gratton et al. identified high aspect ratio nanoparticles as having four-fold increased uptake for HeLa cells in comparison to low aspect ratio particles of similar size and chemistry. The explanation for the difference follows the same reasoning as presented for the rejection hypothesis, but the enlarged surface area affecting high aspect ratio particles is instead theorized to supply beneficial multivalent cationic interactions which promote nonspecific endocytosis. In either case, the morphology of the nanoparticles contributes heavily to their uptake mechanism and represents a major factor in their internalization and clearance potential. Additional shapes such as nonspherical, homogeneous and heterogeneous agglomerates, spherical and tubular micellular capsules, and dendritic structures individually conclude different preferential uptake mechanisms that complicate their ADME profile [22]. Particles that evade phagocytic consumption and remain in circulation for longer periods of time are generally anticipated to possess increased systemic exposure and cytotoxic events, but vast generalizations are inappropriate for nanoparticulate systems due to incongruence among trends in their biocompatibility. Regardless of preexisting

information from similar systems, new nanoparticle preparations necessitate in vitro study to verify the biocompatibility of slight morphological or chemical alterations. Surface charge likewise plays a deterministic role in cellular uptake that rivals the contributions of particle morphology. Both net cationic and anionic charge are correlated with increased toxicity while neutral surfaces are believed to have the greatest biocompatibility. Zwitterionic particles, by contrast, are generally considered benign due to their self-regulated charge balance and have undergone extensive investigation as antimicrobial and antifouling agents. Positively charged nanoparticles express affinity toward the negatively charged phospholipid heads populating the lipid bilayer and encourage endocytosis. Once internalized, the cationic surface charge acts as a proton sponge that disrupts normal lysosomal activity. Negatively charged particles, by comparison, display greater potency in breaching the skin barrier via charge density and have the potential to signal coagulation cascades. Under sufficient doses, anionic nanoparticles can induce thrombosis and eventual embolism. In a similar manner, cationic particles assemble platelet aggregates known as “coronas” that disguise their unmasked chemistry and provide an alternative biological identity. Further complicating the functionality of a singular idealized “crown” covering the nanoparticles, protein adhesion to the particle surface operates by competitive affinity which changes the protein mixture surrounding the nanoparticle and modifies its new biological identity over time [24]. The flux of adsorbed proteins forming the “soft” corona is regulated by a continual state of affinity competition termed the Vroman effect. The presence of a shielding corona can reduce toxicity by preventing the nonspecific cellular internalization of cationic particles and offsetting their membrane disruption and hemolytic capacities or increase toxicity by denaturing attached proteins and generating aberrations that elicit immunogenic and inflammatory responses. Mimicking the myriad of proteins present in biological milieu for in vitro experimentation poses a significant challenge and comprises one of the major sources of disparity between in vitro and in vivo toxicity assessments.

The chemical composition of nanoparticles governs their interaction with cells and milieu and shapes their capacity for oxidative stress production. Applying comparable size regimes, particles of varying fundamental compositions, especially in the case of metal oxide nanoparticles, display significantly different toxicity profiles due to alterations in their base constituencies. Administered metallic nanoparticles are prone to dislodging toxic metal ions in the presence of fluctuating pH zones throughout the body, and the ions’ circulation into accumulation sites such as the liver and kidney present concerns for eventual genotoxic and cytotoxic effects. Iron oxide and copper oxide nanoparticles pose as Fenton or Fenton-like catalysts for radical generation that contribute to lipid peroxidation and deoxyribonucleic acid (DNA) cleavage; gold, highly sought after as photothermal therapy and contrast agents, silver, traditionally used in antimicrobial prophylaxis, and zinc oxide, find use in a variety of applications ranging from filters to food additives, possess dosedependent cytotoxicity; aluminum oxide and titanium dioxide, both employed in polymer and pharmaceutical industries, were initially considered inert but have since garnered attention as oxidative stress and inflammation promoters. Nanosized silica and carbon-based nanomaterials (e.g., carbon nanotubes, fullerenes, carbon black) constitute over 50% of airborne nanomaterials and have extensive literature characterizing their metabolic resistance and size-dependent cytotoxicity. Polymeric nanoparticles propose additional considerations for their toxicity analysis, namely whether the nanoparticles are liable to degrade within the body and whether their metabolites are biocompatible. Although metallic nanoparticles hold similar concerns for metabolic degradation, therapeutic polymer-based nanoparticles often have the designed caveat of undergoing hydrolysis and breaking into their base monomers or analogs. The feature allows on-demand release of medicinal agents into localized delivery sites to enhance the bioavailable fraction

of therapeutics at the site of action while minimizing systemic circulation of drugs with deleterious side effects. Careful engineering is, however, required to ensure toxic degradants are prevented from entering the bloodstream and wreaking havoc downstream of the material's degradation cue. The interplay of balancing the therapeutic and toxic potential of nanoparticles with their metabolites defines a foundational concern for minimizing ROS and inflammation production from therapeutic administration and environmental bioaccumulation, and their evaluation through in vitro testing marks a prerequisite study for any nanoparticle-based treatment.

Characterization and Toxicity Analysis:

To properly evaluate the safety and impact of a nanomaterial, the fundamental physiochemical properties of the material and its interaction and impact upon living cellular systems must be understood. In this section, the most common methods to characterize nanomaterial properties and a set of in vitro toxicity screenings used to determine material safety are presented. The approaches presented herein do not represent the entirety of all available tools and methods but rather a sufficiently broad initial assessment for the evaluation of a new nanomaterial.

Size and Surface Charge:

Evaluation Numerous analytical techniques are available to characterize the toxicological aspects of nanoparticles, but two methods in particular are regularly used to grant critical quantitative information: dynamic light scattering (DLS) and zeta potential (ZP) analysis. With DLS, the random movements of dilute nanoparticles dispersed in solution caused by Brownian motion are detected by monitoring Rayleigh or Mie scattering generated from a monochromatic laser. The intensity fluctuations recorded by the detector are translated into an autocorrelation function which tracks the intensity decay as a function of time. A representative image displaying the translation of scattering intensity into an autocorrelation function is displayed in Fig. 3. In the simplest case, the autocorrelation function is fit to an exponential decay whereby the translational diffusion coefficient is calculated in accordance with the wave vector relating the angle of excitation used by the DLS instrument. The diffusion coefficient is used in conjunction with the Stokes-Einstein equation to estimate the hydrodynamic radius of the particles assuming that the nanoparticles' morphology is confined to a sphere. Averaging the decay rate prior to evaluating the diffusion coefficient yields the ensemble translational diffusion coefficient which is applied to determine the commonly reported z-average nanoparticle diameter. Two primary analytical methods succinctly define the relationship between the autocorrelation function and the sample size distribution: the cumulant and CONTIN algorithms [36]. The cumulant algorithm fits the beginning of the autocorrelation function to a single exponential decay wherein the first cumulant term defines the z-average diameter and the second term indicates the polydispersity index (PDI). The PDI marks the homogeneity of a sample on a zero to one scale; a small PDI ($\leq$0.1) is representative of a monodisperse sample while a large PDI (>0.4) designates a highly polydisperse sample. The CONTIN algorithm employs a broader fitting timescale to isolate size distributions for multiple heterogeneous peaks in polydisperse samples. Modern DLS instruments apply both methods to provide the user with the z-average diameter, PDI, and heterogeneous peak distributions simultaneously. If the particles lack a spherical geometry and Mie scattering dominates, sample anisotropy will noticeably alter the calculated z-average diameter as a function of the irradiation angle used for the sample analysis. Post-measurement analysis can take advantage of equations derived for estimating the sample's rotational diffusion coefficient together with its translational diffusion coefficient to approximate the aspect ratio of rod-like particles [38, 39]. Zeta potential identifies the apparent surface charge of nanoparticles and is often a complementary capability of DLS systems. Particles dispersed in solution innately attract a closely packed layer of oppositely charged molecules to their surface known as the Stern layer, and the Stern layer is

further surrounded by an ionically mixed diffuse layer extending outward toward a hypothetical boundary termed the slipping plane. Together, the Stern layer and slipping plane represent the electric double layer (EDL) enveloping a nanoparticle. As shown in Fig. 4, the electric potential difference between the outermost slipping plane of the EDL and the potential of the dispersant comprises the zeta potential of a colloid. The measured ZP consequently does not signify the surface charge at the nanoparticle–Stern layer interface; the measured potential difference atop the EDL is substantially lower than the potential difference between the surface of the particle and the surrounding medium.

The decay of electrostatic force follows Debye's law as an inverse exponential, and the true surface potential, called the Nernst potential, is only attainable through theoretical approximation. The ZP is determined by correlating the speed of particles in transit to the device's anode or cathode to the magnitude of an externally applied electric field. The velocity is assessed by monitoring the Doppler shift, which relates the frequency change between incident light shown onto electrically mobile particles and a reference laser to the velocity of the colloids in the medium. The electrophoretic mobility of the nanoparticles is directly calculated from the experimentally determined particle velocity and electric field strength, and the mobility is thereafter related to the desired ZP with the Helmholtz–Smoluchowski equation for large particles (>100 nm) having relatively small EDLs with respect to their size or the Hückel equation for small particles (≤100 nm) with comparatively large EDLs. The Helmholtz–Smoluchowski equation is viable for aqueous solutions with high salt concentrations (~10−2 M) while the Hückel equation requires minimal salt interference floating bodies begins to dominate. The magnitude of the measured ZP, therefore, does not provide a conclusive verification of colloid stability; ZP analyzes only particle surface charge without insight into interparticle attractive forces. Solutions with high ZP (±30 mV) are often conferred stability while low ZP solutions (≤10 mV) are considered unstable, but the scale of attractive van der Waals forces can (rarely) revoke traditional classifications. The solution ionic strength also contributes to the nanoparticles' stability by compressing the EDL with increasing ionic strength and lowering the measured ZP.

As mentioned earlier in this chapter, a particle's calculated ZP and, by extension, surface charge play a significant role in deducing their possible toxicity, advancing integrated DLS-ZP systems as essential instruments for precursor biocompatibility evaluations. Although DLS and ZP act as the primary characterization techniques for nanoparticles before their introduction to in vitro assays, numerous other techniques are available that offer comparable analyses or additional worthwhile information. Common practices include electron microscopy (e.g., transmission electron microscopy (TEM) and scanning electron microscopy (SEM)), laser diffraction, and atomic force microscopy (AFM) for sizing and geometry, Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), surface-enhanced Raman spectroscopy (SERS), and solid-state nuclear magnetic resonance spectroscopy (SSNMR) for composition analysis, and ultraviolet-visible spectroscopy (UVVis) and fluorometry for photonic properties. Employing multiple methods in tandem with DLS and ZP analysis help identify some of the more critical parameters composing nanoparticles' toxicity profiles, and their evaluation in concert with in vitro toxicity assessment can reveal the primary mechanisms generating toxicity that require resolution prior to their use.

Oxidative Stress/Inflammation Assays:

The enhanced reactivity of nanoparticles imposed by their high surface area to volume ratio boosts cellular oxidative stress and promotes intracellular damage. Detection of oxidation events through direct measurement of ROS, indicators of oxidative damage such as protein carbonyl content and genotoxicity, and inflammatory markers reveals the in vitro potential of nanoparticles to produce detrimental cellular oxidants. Generation of ROS within the cytoplasm beyond natural

levels is quantifiable via introduction of ROS sensitive dyes such as the nonionic, nonpolar, membrane-permeable fluorophore 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as described by Keston and Brandt. Upon cellular internalization of nonfluorescent DCFH-DA, the fluorophore is enzymatically hydrolyzed by cytosolic esterases into its nonfluorescent polar analog dichlorofluorescein (DCFH), whereby the analog becomes trapped within the cytosol. Hydroxyl radicals and comparable cellular ROS oxidize DCFH into highly fluorescent dichlorofluorescein (DCF) which is monitored using fluorescence microscopy or flow cytometry. Albeit relatively easy to implement and quantitative, the DCFH-DA method for evaluating ROS content is susceptible to inaccuracy due to nonspecific enzymatic oxidation and photooxidation. Catalase offers possible inhibition for enzymatic oxidation, but the results from DCF assays must, nevertheless, be interpreted with caution to avoid overestimating nanoparticles' ROS generation capability. In a comparable method for analyzing mitochondrial respiration, commercial Seahorse Extracellular Flux (XF) Analyzers measure the oxygen consumption rate (OCR) and energy production potential of cells subjected to chemical inhibitors as markers of mitochondrial oxidative stress. Checking the OCR following incubation with antioxidants and subsequent oxidizers tracks oxidation-induced mitochondrial damage inhibition capacity, and the offset in OCR decline observed by the introduction of a therapeutic agent can signify the efficacy of a treatment.

The carbonyl content of proteins increases in response to oxidation from ROS and provides a general indicator of oxidative damage. Several approaches are available for measuring protein carbonyl levels, but two are particularly notable: tritiated borohydride and 2,4-dinitrophenylhydrazine (DNPH). Borohydride reduces protein carbonyls to alcohols with stable tritium labels detectable by spectrophotometric absorbance at 340 nm . As with other carbonyl reagents, extraneous nucleic acids necessitate removal prior to labeling; streptomycin treatment is typically recommended as a pretreatment to precipitate nucleic acids. Employing DNPH as the carbonyl reagent offers a nonradiochemical labeling method, but larger quantities of sampled protein are required for successful analysis. Reaction of the protein carbonyl group with DNPH generates a stable 2,4-dinitrophenyl (DNP) hydrazone product that is detected from its maximum absorbance between 360 and 390 nm and translated to a carbonyl content value using a molar absorption coefficient of 22,000 M−1 cm−1. Both assays produce identical values for the evaluated carbonyl content assuming protein-bound chromophores with absorbances in the range of the applied reagent are subtracted via a blank. The sensitivity and specificity of the DNPH assay is further improved while eliminating the expenditure of excess sample by applying highperformance liquid chromatography (HPLC) or Western blotting with a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system. Immunoassays in the form of ELISA and slot blotting can likewise be applied as highly sensitive techniques for analyzing carbonyl content, but the approaches are less common than the standard DNPH and borohydride methods. Genotoxicity assays survey DNA and chromosomal damage and gene mutations occurring as a consequence of toxin-induced oxidative stress. The comet, micronucleus, Ames, and chromosome aberration assays are comprehensively reviewed by Golbamaki et al. for metal oxide and silica nanomaterials and will be related for their general use herein. The single-cell gel electrophoresis, or comet, assay is a routine method for quantifying DNA breaks using fluorescence microscopy. Under alkaline electrophoresis conditions, supercoiled loops of DNA straighten into tails resembling comets, and the comet's head-totail distance reveals the number of DNA breaks in the sample. The Olive tail moment (OTM), first described by Olive et al., relates the amount of damage in a standard format by reporting the comet tail's length multiplied by its DNA encapsulation percentage. Micronucleus (MN) assays regularly utilize cytochalasin B under the direction of the Organisation for

Economic Co-operation and Development (OECD) Test Guideline no. 487 to test chromosomal breakage by preventing daughter cell separation following mitosis.

Visualization of binucleate cells using fluorescent stains like acridine orange permits quantification of micronucleus frequency among treated mitotic cell populations [80]. The Ames test uses Salmonella (S. typhimurium) and Escherichia coli (E. coli) bacterial strains to test for amino acid production among genetically defunct bacteria as prescribed by OECD Test Guideline no. 471. In the test, mutated bacteria that are incapable of producing an essential amino acid are incubated with the tested substance and the growth of revertant bacteria that regain the ability to synthesize the amino acid is observed. Implementation is rapid and facile but often considered inappropriate for nanoparticles due to the materials' meager uptake by bacterial cells. The chromosome aberration test monitors chromosomal repair process malfunctions by halting mammalian cell cycles using a metaphase-arresting substance as per OECD Test Guideline no. 473 and registering metaphase chromosome aberrations microscopically. The test is limited to clastogen identification and is, therefore, considered inferior to MN assays that also allow for aneugen detection. Lipid peroxidation presents another quantifiable effect of ROS overproduction through evaluation of two major secondary peroxidation products in malondialdehyde (MDA) and 4- hydroxyl-2-nonenal (4-HNE). The more mutagenic of the products, MDA, arises from the decomposition of large polyunsaturated fatty acids (PUFAs) and the metabolism of arachidonic acid (AA) during thromboxane A2 synthesis. Reaction of the by-product with thiobarbituric acid (TBA) at pH 3.5 forms a MDA-TBA adduct that is detected fluorescently at 553 nm with an excitation of 515 nm or spectrophotometrically at 532 nm as part of the thiobarbituric acid reactive substances (TBARS) assay. The assay has received intense scrutiny for its lack of specificity; TBA reacts with a number of substances other than MDA ranging from oxidized lipids to urea, altogether producing drastic overestimations of ROS lipid peroxidation. Efforts to raise the specificity of the assay include using HPLC to isolate the desired MDA-TBA adduct and eliminate background signals from conflicting reactive species.

The more cytotoxic lipid peroxidation product, 4-HNE, occurs as a by-product of AA decomposition and enzymatic and nonenzymatic PUFA peroxidation. The compound's high reactivity toward primary amines to form Schiff bases and thiol or amino compounds to make Michael adducts enables detection using HNE-protein adduct ELISA assays [88]. In samples containing both MDA and 4-HNE, the TBA assay approach described for MDA analysis normally assembles fluorescent adducts for both peroxidation markers due to the assay's nonspecific nature, but substitution of TBA with 1-methyl-2-phenylindole in a hydrochloric acid reaction medium has been shown to promote MDA adduct yields over 4-HNE. Despite its misgivings, the TBARS assay remains the predominant method for determining toxin lipid peroxidation and a viable approach for nanoparticles' peroxidation proclivity.

Endotoxin Assays:

A common challenge for nanoparticles arises from their ability to absorb ambient contaminants to their surface, which can result in an enhanced inflammatory response over what would be expected if the absorbed chemicals were simply free in solution. This is most commonly seen in the case of endotoxins that cause acute inflammatory responses in humans. Endotoxins, or lipopolysaccharides (LPS), are environmentally prevalent pyrogens found in the outer cell wall of gram-negative bacteria that elicit inflammatory cytokines following activation of the coagulation signaling cascade in mammalian systems. The particular cascade sequence discovered for the analogous Atlantic horseshoe crab (Limulus polyphemus) by Bang capitalizes upon the crabs' lethal intravascular coagulation when exposed to Vibrio endotoxin, and the reaction was developed into a routine series of in vitro assay procedures identified under the umbrella of the Limulus

amebocyte lysate (LAL) assay using the blood cells (amebocytes) of the Limulus polyphemus or the homologous Japanese horseshoe crab (Tachypleus tridentatus). Three main assays stem from the LAL approach: the gel clot assay, the coagulogen-based (turbidity) assay, and the chromogenic assay. The gel clot method employs endotoxin-activated enzymatic coagulogen cleavage by combining a portion of LAL solution with the endotoxin sample solution and checking for clotting following sufficient incubation. The technique is somewhat subjective due to confirmation arising from a simple positive or negative tube inversion to visualize clot formation, and quantitation requires inference from serial dilutions. Coagulogen-based assays vary in their quantitation by examining either changes in turbidity, coagulogen reduction during clotting, or peptide fragmentation.

Chromogenic approaches substitute chromogens for coagulogen that release chromophores when cleaved. Traditionally, paranitroaniline is attached to an amino acid sequence resembling the clotting enzyme cleavage site in LAL and colors the solution upon liberation. Absorbance at 405 nm indicates the available concentration of clotting enzyme, which, in turn, reveals the concentration of endotoxin. Chromogenic LAL assays are included in commercial kits such as the Endosafe®-PTS (Portable Test System) for routine endotoxin analysis. In an alternative approach to using the blood of horseshoe crabs for assays, recombinant Factor C (rFC), the priming agent for coagulation, has developed into a standard reagent for endotoxin detection as a method for improving the sensitivity of LAL assays and sparing horseshoe crabs from endangerment. Isolation and implementation of endotoxin-sensitive rFC eliminates possible false-positive reads from β-glucan contamination in LAL assays by eliminating glucan-reactive factor G that acts as a secondary clotting cascade pathway.

Additional methodologies have explored using immunoassays and rabbit pyrogen as alternatives to the popular LAL and rFC assays, but insensitivity and difficulty quantifying biological activity have curtailed their use in comparison to bioassays. Notably, evidence has demonstrated that nanoparticles can perturb LAL assay approaches and disrupt their accuracy, which has generated interest in identifying alternative routes for endotoxin detection to accommodate nanoparticles. Advances include using toll-like receptor 4 (TLR4) as a reporter protein, and the technology has made its way into commercial kits like the HEK-Blue™ detection system.

Evaluating In Vivo toxicity of NMs:
Biodistribution and circulation:

Suitable in vivo detection methods of NMs include radioactive tracing, ICP-MS, atomic absorption spectroscopy, and MRI. The radioactive tracing technique, having the advantages of high sensitivity, credibility, and freedom from interference, has been widely used to obtain information about the behavior of NMs in vivo. By using GO radiolabeled with 188Re, the radioactivity of each tissue has been measured with a gamma-ray counter to demonstrate the pharmacokinetics of GO in mice (Zhang et al. 2011). This strategy has been applied to many NMs. Using a similar method, researchers have uncovered the distribution and other characteristics of a variety of carbon NMs, including C60 (Xu et al. 2007), SWCNT (Yang et al. 2007b), MWCNT (Gao et al. 2011).

ICP-MS is also used to determine the metal NM content and to analyze its biodistribution and blood circulation (Jain et al. 2008). Using atomic absorption spectroscopy, researchers have investigated the biodistribution of GNPs and Au/SiO2 and the circulation kinetics of GNPs in the bloodstream of rabbits (Terentyuk et al. 2009). Additionally, MRI results have revealed the distribution of iron oxide agents (Muldoon et al. 2005). Furthermore, real-time intraoperative NIR fluorescence imaging provides highly sensitive and real-time images, enabling monitoring of the translocation and distribution of NMs (Choi et al. 2010).

Immune Responses and organ Functions:

The immunotoxicity of traditional drugs has been evaluated by using the local lymph node assay (LLNA) and plaque-forming cell (PFC) assay (Jack 1997). However, the different portals of entry inevitably enable NMs to target different populations of cells (Dobrovolskaia and McNeil 2007) and, thus, the standard LLNA test is not recommended for NMs. The lymph node proliferation assay has been used to predict drug immunotoxicity in humans (Weaver et al. 2005) and is recommended for NMs. The effects of NMs on the immune system may be well predicted by the PFC assay. Reticuloendothelial uptake and tests of macrophage function are also useful methods for evaluating NMs (Dobrovolskaia et al. 2009). The systemic immunological responses of mice injected with CNTs containing impurities were revealed by monitoring changes in peripheral T-cell subset and peripheral cytokine levels (Koyama 2009).

In this study, sampled blood was FACS-sorted to separate CD4 and CD8 T lymphocytes, and then nine different cytokines related to inflammatory reactions were measured by ELISA. In assessing organ toxicity, histologic examination is a well-accepted method. The level of oxidative stress is also a toxicity indicator in organs. Both assays can be considered conventional and all-purpose strategies for evaluating organ and tissue toxicity. Corresponding assays should be used to assess effects within specific organs. For example, liver function tests including ALP, alanine transaminase, and aspartate transaminase were used to assess the liver's function (Sahu 2009). The lungs of vehicle- and particle-exposed rats have been assessed by using bronchoalveolar lavage fluid biomarkers, oxidant and glutathione endpoints, and airway and lung parenchymal cell proliferation methods and histopathological evaluation (Sayes et al. 2007). The results of complete blood count tests may reflect the NMs' influence on blood circulation (Schipper et al. 2008).

Conclusion:

Despite similarities in size, nanoparticles threaten multimodal forms of toxicity to unfortunate hosts through numerous mechanisms. Nanomaterial toxicity occurs due to the physicochemical properties of the material determining its proclivity to interact with the various proteins and cells comprising the biological milieu. Catalyzing ROS generation forms one of the most potent contributors to nanoparticles' toxicological components and sparks cascades of oxidative stress and inflammatory signals that ultimately lead to necrosis, expedited apoptosis, or carcinogenesis. The roots of the disastrous cascades are uncovered by observing the interplay between the particles' size and morphology and their chemical composition and apparent surface charge. Evaluation of each property is required to fully understand the toxicity profile of the queried material, and, even with exhaustive characterization, causes for toxicity can be shrouded by the complexity of biological systems. No individual gold standard exists for foreshadowing the toxicity of a unique nanomaterial; rather, combinations of several techniques are necessary to adequately describe the material's toxicological profile. Albeit ubiquitous, sizing from DLS does not provide an insurmountable threshold to bar cellular infiltration and epithelial barrier penetration, and ZP relates only the particle's apparent surface charge. Deduction of cellular uptake, protein corona formation, catalysis potential, and protein denaturation proficiency from routine nanoparticle analysis is, at best, inferential and necessitates in vitro study for verification Akin to the limitations plaguing characterization of physiochemical properties, in vitro assays also lack a one-size-fits-all method for exposing evidence regarding the toxicity or biocompatibility of tested nanoparticles. The myriad of cell types and assays impose a formidable array of tests for identifying individual markers of toxicity, but none of the studies provides an ideal pass-or-fail qualification. Multiple cell types should ideally be screened to replicate the heterogeneity of in vivo conditions, and singular signs of apparent inertness do not

exclude alternative toxicological mechanisms from remaining in play. Combinations of multiple assays are, therefore, needed to sufficiently elucidate physiological responses to the nanoparticle system in question. Issues still persist for several of the established in vitro assays, namely: a method for the parallel high-resolution imaging and precise quantification of nanoparticle internalization beyond the sacrifices present for IFC has yet to be drawn into a standalone instrument; protein-based catalysis assays frequently assume a risk of cross-reactivity with secondary analytes that can mar data from the assays; and the enhanced reactivity of nanoparticles can pre-emptively oxidize analytical reagents or denature proteins that constitute the basis of numerous in vitro approaches. Resolution to the analytical and application-based problems involved with in vitro assays, particularly those associated with nanoparticulate systems, embodies the focus of future endeavors into nanoparticle toxicity analysis.

References:

1. Eifler AC, Thaxton CS (2011) Nanoparticle Therapeutics: FDA Approval, Clinical Trials, Regulatory Pathways, and Case Study In: Hurst SJ (ed) Biomedical Nanotechnology: Methods and Protocols. Humana, Totowa, NJ, pp 325–338. 10.1007/978-1-61779-052-2_21
2. Priyadarsini S, Mukherjee S, Mishra M (2018) Nanoparticles used in dentistry: a review. J Oral Biol Craniofac Res 8(1):58–67. 10.1016/j.jobcr.2017.12.004
3. Copéret C, Héroguel F (2017) Recent advances in surface organometallic chemistry In: Cornils B, Herrmann WA, Beller M, Paciello R (eds) Applied homogeneous catalysis with organometallic compounds. Wiley-VCH Verlag GmbH & Co, Weinheim, pp 1069–1084.
4. Yan N, Xiao C, Kou Y (2010) Transition metal nanoparticle catalysis in green solvents. Coord Chem Rev 254(9):1179–1218.
5. Weiss J, Takhistov P, McClements DJ (2006) Functional materials in food nanotechnology. J Food Sci 71(9):R107–R116.
6. Contado C (2015) Nanomaterials in consumer products: a challenging analytical problem. Front Chem 3:48
7. Raj S, Jose S, Sumod US, Sabitha M (2012) Nanotechnology in cosmetics: opportunities and challenges. J Pharm Bioallied Sci 4 (3) :186–193.
8. Boxberg F, Tulkki J (2004) Quantum dots: phenomenology, photonic and electronic properties, modeling and technology the handbook of nanotechnology. Nanometer structures: theory, modeling, and simulation. SPIE Press, Bellingham
9. Akbarzadeh A, Samiei M, Davaran S (2012) Magnetic nanoparticles: preparation, physical properties, and applications in biomedicine. Nanoscale Res Lett 7(1):144–144.
10. Smulders S, Luyts K, Brabants G, Landuyt KV, Kirschhock C, Smolders E, Golanski L, Vanoirbeek J, Hoet PHM (2014) Toxicity of nanoparticles embedded in paints compared with pristine nanoparticles in mice. Toxicol Sci 141(1):132–140.
11. Clichici S, Filip A (2015) In vivo assessment of nanomaterials toxicity. Nanomaters toxicity and risk assessment.
12. Vasilakes AL, Dziubla TD, Wattamwar PP (2013) Polymeric nanoparticles In: Bader RA (ed) Engineering polymer systems for improved drug delivery. John Wiley & Sons, Inc., pp 117–161.
13. Cabral H, Matsumoto Y, Mizuno K, Chen Q, Murakami M, Kimura M, Terada Y, Kano MR, Miyazono K, Uesaka M, Nishiyama N, Kataoka K (2011) Accumulation of sub-100 nm polymeric

micelles in poorly permeable tumours depends on size. Nat Nanotechnol 6:815

14. Perrault SD, Walkey C, Jennings T, Fischer HC, Chan WCW (2009) Mediating tumor targeting efficiency of nanoparticles through design. Nano Lett 9(5):1909–1915.
15. Jiang W, Kim BYS, Rutka JT, Chan WCW (2008) Nanoparticle-mediated cellular response is sizedependent. Nat Nanotechnol 3:145
16. Jain RK, Stylianopoulos T (2010) Delivering nanomedicine to solid tumors. Nat Rev Clin Oncol 7:653
17. Caldorera-Moore M, Guimard N, Shi L, Roy K (2010) Designer nanoparticles: incorporating size, shape and triggered release into nanoscale drug carriers. Expert Opin Drug Deliv 7 (4) :479–495.
18. Gratton SEA, Ropp PA, Pohlhaus PD, Luft JC, Madden VJ, Napier ME, DeSimone JM (2008) The effect of particle design on cellular internalization pathways. Proc Natl Acad Sci 105 (33):11613– 11618.
19. Huo S, Jiang Y, Gupta A, Jiang Z, Landis RF, Hou S, Liang XJ, Rotello VM (2016) Fully zwitterionic nanoparticle antimicrobial agents through tuning of core size and ligand structure. ACS Nano 10(9):8732–8737.
20. Fleischer CC, Payne CK (2014) Nanoparticle–cell interactions: molecular structure of the protein corona and cellular outcomes. Acc Chem Res 47(8):2651–2659.
21. Vroman L, Adams AL (1969) Identification of rapid changes at plasma–solid interfaces. J Biomed Mater Res 3(1):43–67.
22. Owens DE, Peppas NA (2006) Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int J Pharm 307 (1):93–102.
23. Li S-D, Huang L (2010) Stealth nanoparticles: high density but sheddable PEG is a key for tumor targeting. J Control Release 145 (3) :178–181.
24. Borm, P. J. A., and W. Kreyling. 2004. Toxicological hazards of inhaled nanoparticles—potential implications for drug delivery. Journal of Nanoscience and Nanotechnology**.** 4:521–531.
25. Choi, A., S. J. Cho, J. Desbarats, J. Lovric, and D. Maysinger. 2007a. Quantum dot-induced cell death involves Fas upregulation and lipid peroxidation in human neuroblastoma cells. Journal of Nanobiotechnology 5:1.
26. Franzen, S., A. G. Tkachenko, H. Xie et al. 2004. Cellular trajectories of peptide-modified gold particle complexes: Comparison of nuclear localization signals and peptide transduction domains. Bioconjugate Chemistry 15:482–490.
27. Gharbi, N., M. Pressac, M. Hadchouel et al. 2005. [60]Fullerene is a powerful antioxidant in vivo with no acute or subacute toxicity. Nano Letters 5:2578–2585.
28. Ghibelli, L., M. De Nicola, D. M. Gattia et al. 2007. Effect of different carbon nanotubes on cell viability and proliferation. Journal of Physics: Condensed Matter 19:395013.
29. Girard, D., D. M. Goncalves, and S. Chiasson. 2010. Activation of human neutrophils by titanium dioxide (TiO(2)) nanoparticles. Toxicology In Vitro 24:1002–1008.
30. Gratton, S. E. A., P. A. Ropp, P. D. Pohlhaus et al. 2008. The effect of particle design on cellular internalization pathways. Proceedings of the National Academy of Sciences 105:11613–11618.
31. Gref, R., Y. Minamitake, M. T. Peracchia et al. 1994. Biodegradable long-circulating polymeric nanospheres. Science 263:1600.

32. Huff, T. B., M. N. Hansen, Y. Zhao, J. X. Cheng, and A. Wei. 2007. Controlling the cellular uptake of gold nanorods. Langmuir 23:1596–1599.

33. Hussain, S. M., L. K. Braydich-Stolle, A. M. Schrand et al. 2009. Toxicity evaluation for safe use of nanomaterials: Recent achievements and technical challenges. Advanced Materials 21:1549–1559.

34. Ishiyama, M., H. Tominaga, M. Shiga et al. 1995. Novel cell proliferation and cytotoxicity assays using a tetrazolium salt that produces a water-soluble formazan dye. Toxicology In Vitro. 8:187–190.

35. Chakravarty, P., R. Marches, N. S. Zimmerman et al. 2008. Thermal ablation of tumor cells with antibody-functionalized single-walled carbon nanotubes. Proceedings of the National Academy of Sciences 105:8697–8702.

36. Fernandez-Suarez, M., and A. Y. Ting. 2008. Fluorescent probes for super-resolution imaging in living cells. Nature Reviews Molcular Cell Biology 9:929–943.

37. Chan, W. C. W., B. D. Chithrani, and A. A. Ghazani. 2006. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. Nano Letters 6:662–668.

38. Iwai, N., and H. Yamawaki. 2006. Cytotoxicity of water-soluble fullerene in vascular endothelial cells. American Journal of Physioliogy – Cell Physiology 290:C1495–C1502.

39. Jack, H. D. 1997. Issues with introducing new immunotoxicology methods into the safety assessment of pharmaceuticals. Toxicology 119: 95–101.

40. Jain, T. K., M. K. Reddy, M. A. Morales, D. L. Leslie-Pelecky, and V. Labhasetwar. 2008. Biodistribution, clearance, and biocompatibility of iron oxide magnetic nanoparticles in rats. Molecular Pharmaceutics 5:316–327.

41. Ji, Z. Q., H. Sun, H. Wang et al. 2006. Biodistribution and tumor uptake of C60(OH)x in mice. Journal of Nanoparticle Research 8:53–63.

42. Cho, W. S., M. Cho, J. Jeong et al. 2009. Acute toxicity and pharmacokinetics of 13 nm-sized PEG-coated gold nanoparticles. Toxicology and Applied Pharmacology 236:16–24.

43. Kam, N. W. S., and H. Dai. 2005. Carbon nanotubes as intra- cellular protein transporters: Generality and biological functionality. Journal of the American Chemical Society 127:6021–6026.

44. Kam, N. W. S., Z. Liu, and H. Dai. 2005. Functionalization of carbon nanotubes via cleavable disulfide bonds for efficient intracellular delivery of siRNA and potent gene silencing. Journal of the American Chemical Society 127: 12492–12493.

45. Kim, J. S., T. J. Yoon, K. N. Yu et al. 2006. Toxicity and tissue distribution of magnetic nanoparticles in mice. Toxicological Sciences 89:338.

46. Koopman, G., C. P. M. Reutelingsperger, G. A. M. Kuijten et al. 1994. Annexin-V for flow cytometric detection of phosphatidylserine expression on B-cells undergoing apoptosis. Blood 84:1415–1420.

47. Carlson, C., S. M. Hussain, A. M. Schrand et al. 2008. Unique cellular interaction of silver nanoparticles: Size-dependent generation of reactive oxygen species. Journal of Physical Chemistry B 112:13608–13619.

48. Lockman, P. R., J. M. Koziara, R. J. Mumper, and D. D. Allen. 2004. Nanoparticle surface charges alter blood–brain barrier integrity and permeability. Journal of Drug Targeting 12:635–641.

49. Lovric, J., S. J. Cho, F. M. Winnik, and D. Maysinger. 2005. Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death. Chemistry & Biology 12:1227–1234.

50. Foldbjerg, R., P. Olesen, M. Hougaard et al. 2009. PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. Toxicology Letters 190:156–162.

51. Manna, S. K., S. Sarkar, J. Barr et al. 2005. Single-walled carbon nanotube induces oxidative stress and activates nuclear transcription factor-kappaB in human keratinocytes. Nano Letters 5:1676–1684.

52. Matsumoto, K., C. Sato, R. L. D. Whitby, and N. Shimizu. 2009. Carbon nanotubes stimulate neurite outgrowths of neurons by activation of MAPK/ERK signal transduction. New Biotechnology 25:S27–S28.

53. Mitchell, L., F. Lauer, S. Burchiel, and J. McDonald. 2009. Mechanisms for how inhaled multiwalled carbon nanotubes suppress systemic immune function in mice. Nature Nanotechnology 4:451–456.

54. Hauck, T. S., A. A. Ghazani, and W. C. W. Chan. 2008. Assessing the effect of surface chemistry on gold nanorod uptake, toxicity, and gene expression in mammalian cells. Small 4:153–159.

55. Mitragotri, S., J. A. Champion, and Y. K. Katare. 2007. Making polymeric micro- and nanoparticles of complex shapes. Proceedings of the National Academy of Sciences of the United States of America 104:11901–11904.

56. Gajdosíková, A., A. Gajdosík, M. Koneracká et al. 2006. Acute toxicity of magnetic nanoparticles in mice. Neuroendocrinology. Letters Supplement 2:96–99.

57. Mooney, E., P. Dockery, U. Greiser, M. Murphy, and V. Barron. 2008. Carbon nanotubes and mesenchymal stem cells: Biocompatibility, proliferation and differentiation. Nano Letters 8:2137–2143.

58. Mu, Q., D. L. Broughton, and B. Yan. 2009a. Endosomal leakage and nuclear translocation of multiwalled carbon nanotubes: Developing a model for cell uptake. Nano Letters 9:4370–4375.

59. Gao, N., Q. Zhang, Q. Mu et al. 2011. Steering carbon nanotubes to scavenger receptor recognition by nanotube surface chemistry modification partially alleviates NFκB activation and reduces its immunotoxicity. ACS Nano 5:4581–4591

60. Nakanishi, T., J. Kunisawa, A. Hayashi et al. 1999. Positively charged liposome functions as an efficient immunoadjuvant in inducing cell-mediated immune response to soluble proteins. Journal of Controlled Release 61:233–240.

61. Nan, A., X. Bai, S. J. Son, S. B. Lee, and H. Ghandehari. 2008. Cellular uptake and cytotoxicity of silica nanotubes. Nano Letters 8:2150–2154.

62. Oberdörster, G., E. Oberdörster, and J. Oberdörster. 2005. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. Environmental Health Perspectives 113:823–839.

63. Palmeira, C. M. 2008. In vitro assessment of silver nanoparticles toxicity in hepatic mitochondrial function. EOARD, Unit 4515 BOX 14, APO AE 09421.

64. Pan, X., L. Chen, S. Liu et al. 2008. Antitumor activity of G3139 lipid nanoparticles (LNPs). Molecular Pharmaceutics 6:211–220.

65. Park, T. H., J. A. Kim, N. H. Lee et al. 2011. Enhancement of neurite outgrowth

in PC12 cells by iron oxide nanoparticles. Biomaterials 32:2871–2877.

66. Pernodet, N., X. H. Fang, Y. Sun et al. 2006. Adverse effects of citrate/gold nanoparticles on human dermal fibroblasts. Small 2:766–773

67. Reddy, S. T., A. J. Van Der Vlies, E. Simeoni et al. 2007. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. Nature Biotechnology 25:1159–1164.

68. Ryman-Rasmussen, J. P., M. F. Cesta, A. R. Brody et al. 2009. Inhaled carbon nanotubes reach the subpleural tissue in mice. Nature Nanotechnology 4:747–751.

69. Rzigalinski, B. A., and J. S. Strobl. 2009. Cadmium-containing nanoparticles: Perspectives on pharmacology and toxicology of quantum dots. Toxicology and Applied Pharmacology 238:280–288.

70. Sahay, G., D. Y. Alakhova, and A. V. Kabanov. 2010. Endocytosis of nanomedicines. Journal of Controlled Release 145:182–195.

71. Sahu, S. C. 2009. Hepatotoxic potential of nanomaterials. In Nanotoxicity, Sahu, S. C. and Casciano, D. A., eds., 183–189. Chichester, UK: John Wiley & Sons.

72. Sayes, C. M., J. D. Fortner, W. Guo et al. 2004. The differential cytotoxicity of water-soluble fullerenes. Nano Letters 4:1881–1887.

73. Sayes, C. M., A. A. Marchione, K. L. Reed, and D. B. Warheit. 2007. Comparative pulmonary toxicity assessments of C60 water suspensions in rats: Few differences in fullerene toxicity in vivo in contrast to in vitro profiles. Nano Letters 7:2399–2406.

74. Schipper, M. L., N. Nakayama-Ratchford, C. R. Davis et al. 2008. A pilot toxicology study of single-walled carbon nanotubes in a small sample of mice. Nature Nanotechnology 3:216–221.

75. Schmid, S. L., and S. D. Conner. 2003. Regulated portals of entry into the cell. Nature 422:37–44.

76. Takagi, A., A. Hirose, T. Nishimura et al. 2008. Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube. Journal of Toxicological Sciences 33:105.

77. Terentyuk, G. S., G. N. Maslyakova, L. V. Suleymanova et al. 2009. Circulation and distribution of gold nanoparticles and induced alterations of tissue morphology at intravenous particle delivery. Journal of Biophotonics 2:292–302.

78. Valiyaveettil, S., P. V. AshaRani, G. L. K. Mun, and M. P. Hande. 2009. Cytotoxicity and genotoxicity of silver nanoparticles in human cells. ACS Nano 3:279–290.