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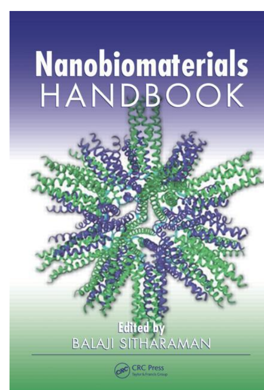


# Nanobiomaterials Handbook

Balaji Sitharaman

# Biocompatibility of Nanomaterials

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# 30

# Biocompatibility of Nanomaterials: Physical and Chemical Properties of Nanomaterials Relevant to Toxicological Studies, In Vitro and In Vivo

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## 30.1 Introduction

Nanomaterials are currently at the center of the materials science and the medicinal worlds. The demand for nanotechnology drives the development of products such as highly effective sunscreens, blood glucose monitors, or light bulbs. This increasing incorporation of nanomaterials into consumer products provides opportunity for increased exposure to the workers who produce the materials, the consumers who utilize the products, as well as the environments into which the product is introduced. The development of safety protocols and the establishment of exposure regulations will naturally play an important role in the development of the field of nanotechnology.

Nanomaterials generally consist of nanofilms, nanoparticles, and nanocomposites. Nanoparticles, in the toxicological sense, are defined as particulates with a primary particle size with one dimension

less than 100 nm. In addition to size, the nanoparticles exhibit novel properties that differ from their bulk, or micron size scale, counterpart (i.e., electronic and/or insulation properties). These novel uses for engineered nanomaterials (ENMs) are often derived from their ability to exhibit monodispersity, large surface area: size ratio, and a high order. Nanoparticles may be synthesized with combinations of various properties to yield unique structural and functional features useful in product development. The resultant nanomaterials may be formulated as dry nanopowder or suspended in either an aqueous or organic solvent. These phases of nanoparticles lead to altered physicochemical characteristics. In addition to the dry and suspended phases, one might also classify nanoparticles as in vivo and ex vivo phases. It has become important to the toxicologist to determine which nanomaterial property, if any, will help to predict a physiological response in each of the four phases.

A list of unique nanomaterial properties would take many hundreds of entries; however, nanotoxicologists have generally defined a subset of characteristics that may quickly and economically be evaluated. This subset of nanomaterial properties, including size, shape, surface area, crystalline state, and surface modification or functionalization, allows insight into the potential toxicological impacts of these novel materials. Each of these characteristics has been shown to influence a corresponding biological interaction. For example, size and surface charge have been shown to influence nanomaterial endocytosis while surface modification and crystalline state are influential factors in cytotoxicity. Predictive measures of toxicity, similar to the structure activity relationship (SAR) of organic compounds, of nanomaterials are needed to allow efficient evaluation prior to use.

## 30.2 Material Characterization Methods

### 30.2.1 Transmission Electron Microscopy and Energy Dispersive Spectroscopy

The use of transmission electron microscopy (TEM) has become a necessary characterization tool in the fields of nanotechnology and nanotoxicology. TEM takes advantage of the extremely small wavelength of electrons (□ 0.2 nm) to increase the resolution and magnification of nanoscale particulates and cellular organelles. In transmission electron microscopy, the specimen is exposed to an electron beam, and subsequently the transmitted electrons are viewed on a phosphorescent screen, a CCD camera, or film. Depending on the nanomaterial characteristics, the TEM can provide information such as size, shape, agglomeration state, and crystalline structure of nanoparticles. In addition, TEM may be used postexposure both in vitro and in vivo to determine the intracellular localization of nanoparticles as well as any cellular structural changes.

While TEM provides a plethora of information surrounding nanomaterial characterization, there are some limitations to this technique. Due to the high energy of the electron beam (i.e., 60–300 keV), biological specimens are rapidly damaged upon exposure. Therefore, proper biological specimen preparation determines the quality of the specimen. Proper preparation is a multistep process consisting of fixation, dehydration, embedding, and sectioning. Further explanation on this process can be found in *Electron Microscopy* by Bazzola and Russel as well as *Biological Specimen Preparation* by Glauert and Lewis. Subsequently, post-staining with a combination of uranyl acetate and lead citrate will enhance contrast and allow for better visualization of the subcellular structure (Reynolds 1963).

As previously mentioned, specimen preparation plays a major role in the quality of data retained from an electron microscope. Nanoscientists must take exceptional precautionary measures when drawing conclusions based solely on TEM images. For example, postfixation with osmium tetroxide ($OsO_4$) may introduce small (1–2 nm) nanoparticles into the specimen. In addition, if samples are poststained, one must be assured that visualized nanoparticles are not artifacts from the protocol itself. Careful analytical techniques, such as those described in the next section, when combined with image analysis are the undefined standards in nanotechnology.

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While TEM may itself be useful as a characterization tool, combinations of TEM with alternative methodologies such as energy dispersive spectroscopy (EDS), electron diffraction (ED), and high-resolution transmission electron microscopy (HRTEM) may be useful for determining additional characterization parameters such as chemical composition and speciation. Electron diffraction is a tool used by materials scientists to determine the crystal structure of a solid particle. When excited electrons pass through the nanoparticles, the electrons produce a signature diffraction pattern. Once this pattern has been attained, it may be compared with that of a standard material. This technique may come into play when a particle with a single composition (i.e., $TiO_2$) has multiple crystalline states, which induce differential amounts of toxicity (Sayes et al. 2006). Similarly, HRTEM may also be used to study the crystallographic structure at the atomic scale.

Energy dispersive spectroscopy as a micro-analytical tool can yield both quantitative and qualitative results when utilized by the trained personnel. When emitted electrons strike the atoms of interest in the sample, inelastic reactions occur that generate emitted electrons and x-rays subsequently detected via spectroscopy. With the advent of powerful computers, these detected signals may then be processed for exact elemental composition. When preparing a specimen for EDS analysis, one must be careful that no additional elements have been added to the specimen that may interfere with the spectral analysis of the element of interest.

An alternative method of electron microscopy has recently been used to identify nanoparticle composition in a heterogeneous exposure. Scanning transmission electron microscopy (STEM) (field emission gun) utilizes a very small electron beam to scan the specimen of interest. STEM emission may be combined with subsequent high-angle annular dark field (HAADF) analysis to provide a reverse contrast image with brightness dependent on atomic number squared. The use of STEM-HAADF as a characterization tool provides the toxicologist with a novel method to determine the composition of individual nanoparticles in a complex matrix (Figure 30.1).

## 30.2.2 Raman and Other Spectroscopies

One of the most common analytical techniques in chemistry is spectroscopy, the science concerned with the measurement and interpretation of electromagnetic spectra arising from either emission or absorption of radiant energy by various chemicals. These spectroscopic techniques are useful for particle analyses. Information about the chemical composition, structure, surface functionality, and optical/electronic properties of the sample can be obtained. Different types of spectroscopic techniques can be used to characterize nanoparticles. For example, mass spectroscopy is used to determine the masses of small electrically charged particles, and can be utilized to measure properties of on a particle surface

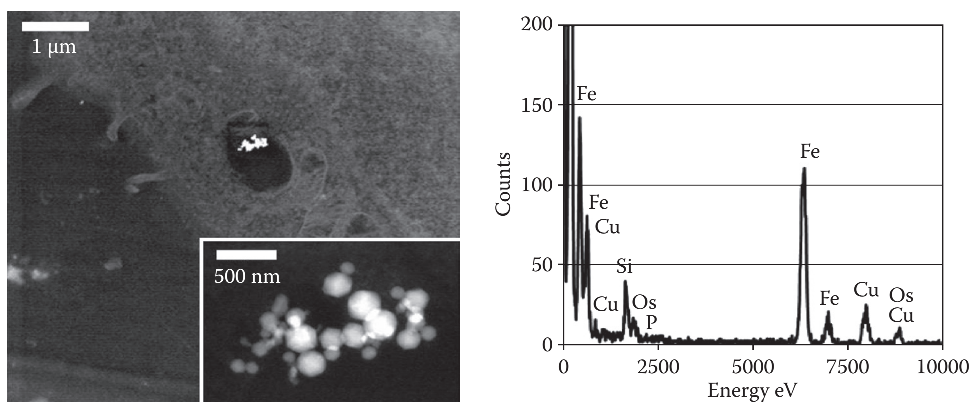


**FIGURE 30.1** STEM image and EDS spectra of human lung epithelial cells exposed to ~30 nm $Fe_2O_3$ nanoparticle 24 h post-exposure.

such as bound (absorbed or adsorbed proteins). Raman spectroscopy is used to study vibrational, rotational, and other low-frequency modes in a system and can be utilized to determine type and degree of functionalization on the sidewall of a carbon nanotube. Absorption spectroscopy is used to quantify the amount of photons a substance absorbs and can be utilized to measure the size of gold nanoshells: absorbance is red-shifted (decreased wavelength) as the thickness of shell increases. Fluorescence spectroscopy is used to analyze the different frequencies of light emitted by a substance, which is then used to determine the structure of the vibrational levels of that substance. In nanoparticle characterization, fluorescence spectroscopy can give information on the functionality of quantum dots. The longer the quantum dot fluoresces, the increased semiconducting effects.

Raman spectroscopy has recently gained popularity for the advanced chemical analysis of surfaces. Raman spectroscopy is a spectroscopic technique used in material science to study vibrational and rotational frequencies in a system. The technique measures shifts in inelastic scattering, or Raman scattering, of light from a visible, near infrared, or near ultraviolet light source. The shift in energy gives information about the material surface characteristics. In nanoscience, Raman spectroscopy is used to characterize surface properties materials, measure temperature, and determine crystallinity. The Raman signal units is a measurement of the ratio between the Stokes (down-shifted) intensity and anti-Stokes (up-shifted) intensity peaks.

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### 30.2.3 Dynamic Light Scattering

Dynamic light scattering (DLS) (also known as Photon Correlation Spectroscopy [PCS]) is used for measuring the nanoparticle size and size distribution when suspended in a medium. In actuality, DLS measures the browning motion of the nanoparticles. Brownian motion is the random movement of suspended particles due to bombardment of the particulate by solvent molecules. As solvent molecules collide with the nanoparticles, they exert a force that is capable of moving the particles in a random direction. This random movement will be much more exaggerated in a small particle than in a large particle of the same composition over the same time period. This movement, otherwise known as the translational diffusion coefficient ($D$), can then be related to the particle's hydrodynamic radius via the Einstein–Stokes Equation 30.1:

$$d(H) = \frac{kT}{3\pi nD} \tag{30.1}$$

Combining $D$ and Boltzmann's constant, $k$, with the physical parameters of the system, including a stable temperature $T$, and the viscosity of the solvent $n$, one might deduce the hydrodynamic radius of the nanoparticle of interest.

DLS is a common instrument in the nanotoxicology laboratory. It provides the researcher with a quick, cost-effective means of generating a size profile of nanomaterials in suspension. DLS output, on a machine such as the Zetasizer NanoZS, displays intensity on the dependent axis and size on the independent. In addition, DLS may also be used to measure biological samples such as proteins when in a biological buffer.

While DLS is widely used in laboratories, one must constantly be aware of its limitations. For example, in a polydisperse sample, the larger particles will scatter light many more times. More specifically, the intensity of scattered light is proportional to the diameter of the particle to the sixth power. Therefore, a 20 nm particle would scatter 64X as much light as a 10 nm particle (64,000,000 versus 1,000,000 U). This property is called Rayleigh scattering. First, it is difficult to measure single particles using light scattering when aggregates or agglomerates are present in the focal suspension, which is a major limitation of the instrument. Therefore, a polydisperse sample mixture may read as if it were void of the smaller fraction of nanoparticles, or simply be unsuitable for analysis via DLS. Second, many nanoparticles

tend to agglomerate once in solution. This agglomeration state may yield particulates in the micrometer ($10^{-6}$) range. This in turn would make it hard to distinguish a particle size on the order of three orders of magnitude lower. Third, DLS measurements should be taken in pristine solutions. For example, a cell culture medium with the addition of fetal bovine serum contains many proteins that interact with the nanoparticle surface. While this alteration in the surface chemistry by the addition of FBS is known to play an important role in toxicology, this protein adsorption would make the nanoparticle size larger due to the increased hydrodynamic radius. In situations where biomolecules are utilized, it is important that control samples be run without the nanoparticle to determine the sizing profile of components in the suspension medium.

### 30.2.4 Zeta Potential

The zeta potential of a nanoparticle represents the charge on the surface in addition to the adsorbed layer of counter ions. The adsorbed layer, hereafter known as the electric double layer, is generated from oriented solute molecules and ions surrounding the nanoparticle surface. The electric double layer is sensitive to various changes in pH and ionic strength. All nanoparticles in suspension carry a net charge. This charge, be it negative, positive, or neutral, is dependent upon the micro environmental conditions of the suspension media. For example, all nanoparticles have an isoelectric point (IEP). This isoelectric point occurs when a particle's zeta potential has zero net charge (IEP: $Z = 0\,mV$). When this occurs in the Nanosizer ZS system, the particle experiences no movement in response to alternating polarity in the test cuvette. Recent work in our lab has shown that the IEP may play a role in the toxicity of nanomaterials (Berg et al. 2009). One hypothesis that has arisen is the fact that the charge at physiological pH may direct certain protein modifications of the nanoparticle surface. This modification may then mediate cellular response.

The zeta potential is also used as a measurement of colloidal stability. A zeta potential value above $\pm 30\,mV$ is considered a stable system. Zeta potential values with an absolute value less than 30 mV are considered unstable systems and are prone to agglomeration. This agglomeration is due to the lack of a charge–charge repulsion between individual nanoparticles.

## 30.3 In-Line Characterization

Nanomaterial characterization normally occurs independently to either in vitro or in vivo exposure. However, this provides many opportunities for variability in an experiment (i.e., nanoparticle synthesis variation, sample contamination). An alternative to independent or off-line sampling would be to couple the nanomaterial characterization to the exposure. This online measurement is performed in series or parallel to the exposure chamber and would allow precise characterization as seen for the test organism. The following methods have been utilized as in-line methods for nanotoxicological exposures in both an aerosolized form and in an aqueous or organic solvent.

### 30.3.1 Differential Mobility Analyzer

Many physicochemical properties are influenced when introduced to an aqueous environment that occurs during in vitro and in vivo testing. As previously mentioned, this change in environmental conditions may lead to an altered agglomeration state. However, engineered nanoparticles, in an aerosolized suspension, are sometimes more monodisperse and may lead to altered toxicity. Determining particle size in an aerosol can be accomplished through the use of a differential mobility analyzer (DMA). Furthermore, the DMA is often coupled with a condensation particle counter (CPC) or an aerosol particle mass analyzer (APM) in order to identify particle size distribution. The use of this measurement parameter allows the correct identification of dose in an aerosolized exposure.

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The DMA is an instrument that can sort ultrafine particles according to size while aerosolized. To begin, DMA applies a charge to particles in the sample. These charged particles are then passed through a chamber consisting of a central rod and an air flow. As the particles pass through this environment, they are separated by their mobility. Mobility is determined by a combination of particle size and electrical charge. It is important to note that in these situations only particles that have been given a positive charge are measured, and those with a negative charge are repelled from the central rod and exit the instrument in the exhaust flow. Electrical mobility, $Z_p$, is dependent upon the average particle diameter, $D_p$; gas viscosity, □□; and the number of charges on the particle, $n$, according to Equation 30.2 (Willeke and Baron 2005; Stevens et al. 2008):

$$Z_p = \frac{neC}{3\pi\mu D_p} \tag{30.2}$$

For extremely small nanoparticles (<~70 nm) the Cunningham Correction, $C$, becomes a necessary addition to the formula to correct nanoparticle drag. Lastly, $e$ is equal to $1.602 \times 10^{-19}$ C.

There remains much debate on the correct method for dosimetry of nanomaterials used for toxicological studies; be it mass, surface area, volume, or particle number. Ostraat et al. (2005) suggests the use of a radial DMA (RDMA). RDMA is the most relevant for nanoparticle classification due to its extremely high transmission efficiency (Ostraat et al. 2005). Developing a complete aerosol exposure system composed of a nanoparticle source in series with a DMA and CPC or APM, remains the most complete way of characterizing nanoparticles, as they are seen in ambient exposure conditions in an aerosolized form (Oyabu et al. 2007). It has also been hypothesized that the physicochemical parameters may be responsible for toxicity; however, for an in vivo exposure, both these properties (i.e., primary particles versus agglomerates) may be explored. For example, nanoparticle agglomerates are not broken up in the DMA; however, they may be extracted prior to analysis through the use of an inertial impactor that would eliminate a large portion of the micron-sized agglomerates prior to introduction to the DMA (Ma-Hock et al. 2007; Scheckman and McMurry 2009). In addition, exposure to primary size nanoparticle may be accomplished through a method that combines synthesis, DMA characterization, and exposure in series.

### 30.3.2 Flow-Field Fractionation

Field-Flow fractionation (FFF) is a separation technique that comprises elements of chromatography with field-driven techniques such as ultracentrifugation and electrophoresis (Giddings 1993). These elution techniques can be used to separate complex mixtures of nanoparticles, macromolecules, and other particulate matter across a broad range of sizes from ~1 nm up to 100 μm. The separation of complex mixtures occurs as the sample mixture (in suspension) passes through a complex field that is oriented perpendicular to the direction of flow. This field applies differential amounts of force on nanoparticles with different properties (i.e., size, density, and charge). However, the field in FFF does not induce the separation of nanoparticles but forces the particulates against the accumulation wall where particulates may then be separated by differential flow rates of particulates with different physicochemical properties.

FFF is comprises multiple techniques that differ in both the field applied and the mechanism of separation. In sedimentation-FFF, particles are driven against a wall as a centrifugal force is applied. Sedimentation-FFF has allowed the detection of unlabeled nanoparticles when extracted from an ex vivo sample, in addition to determining the sizing profiles of those extracted particles (Deering et al. 2008). While sedimentation-FFF may be used to achieve high selectivity among samples, it often has limitations in the range of 10–30 nm due to the high speeds necessary to achieve separation (Giddings 1993).

A more appropriate technique used to elute small nanoparticles (<10 nm) would be flow-FFF. Flow-FFF is a density independent technique (unlike sedimentation-FFF) in which a perpendicular flow drives the nanoparticles against the accumulation wall. Flow-FFF has been used to analyze ~25 nm $TiO_2$ particles in commercial sunscreen (Contado and Pagnoni 2008). In this case, flow-FFF was paired with ICP-AES to generate both the size property and the $TiO_2$ content, measured by the amount of Ti in the sample. Flow-FFF has also been used as a way to monitor the growth of nanoparticles during synthesis (Chen et al. 2005a).

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## 30.4 Characterization In Vitro and In Vivo

While many nanomaterial properties may be readily measured in an acellular environment, the toxicologist strives to determine the response of an organism to such materials. Current nanotoxicology protocols suggest that careful characterization of nanomaterials is performed prior to in vitro or in vivo use. However, as beneficial as these measurements are, many of the nanoparticle properties change when introduced to an aqueous or physiological system. For example, nanomaterials will agglomerate when introduced to a phosphate buffered saline (PBS) or a cell culture media (CCM) sample (Sager et al. 2007; Murdock et al. 2008). Due to the differences in physicochemical properties when suspended in a medium, there has been an attempt to utilize ex vivo analyses such as the vitamin C assay and the hemolytic potential assay to predict nanomaterial toxicity (Warheit et al. 2007b). Furthermore, florescence-based assays, such as the 2′,7′-dichloroflourescin diacetate (DCFH-DA) assay, may be used as an estimation of oxidative stress in an in vitro setting.

### 30.4.1 Vitamin C Assay

The surface of a nanoparticle has been identified as a potential source of leached ions and reactive species. There are a variety of techniques, and an array of colorimetric probes can be used to measure the reactivity of the surface of a nanoparticle. Examples of techniques include zeta potential, isoelectric point, hemolytic potential, and electron spin resonance (ESR) or electron pair resonance (EPR). Examples of probes are the photodegradation of aqueous Congo Red and degradation of Vitamin C.

In regard to identifying and quantifying the reactivity of a particle's surface, it is important to note that there is no single technique that can be used for all nanoparticle types. One example of measuring the surface activity of a material is photocatalytic degradation. Another metric is delta b* using the Vitamin C assay. The Vitamin C Yellowing test has been developed to correlate the surface of a nanoparticle, such as $TiO_2$, to its chemical stability. Available evidence indicates that the mechanism for color development in the test is the formation of a charge transfer complex between ascorbic acid 6-palmitate and the "active sites" on the ultrafine $TiO_2$ surface (Rajh et al. 1999; Warheit et al. 2007b). The redox reaction of ascorbic acid is shown in Figure 30.2.

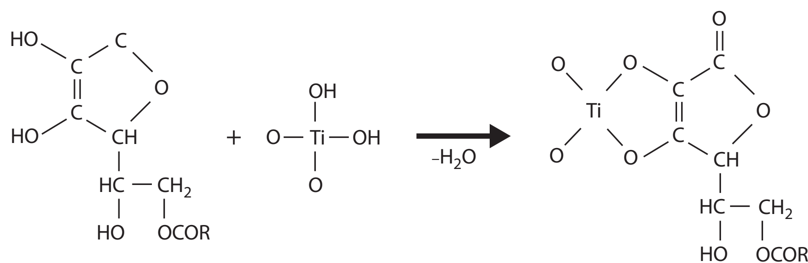


**FIGURE 30.2** Proposed structure of $TiO_2$ nanoparticle with ascorbic acid 6-palmitate.

### 30.4.2 Hemolytic Potential

The hemolytic assay provides a rapid and cost-effective way of determining the effects of particles on a biological membrane. The particulate assay has been used in the past century with a variety of particulates including silicate powders, asbestos, and, more recently, a multitude of nanoparticles (Harington et al. 1971; Nolan et al. 1981; Warheit et al. 2007). Hemolysis (i.e., rupturing of erythrocytes) is an important ex vivo characterization method that may be used in addition to assessing cell death. The ability of nanoparticles to act on a biological membrane is extremely important as such particles are incorporated into medicinal devices for both therapeutic and imaging purposes.

The erythrocyte provides a unique model as a biological membrane system. During erythropoesis, the mammalian erythrocyte extrudes the nucleus and organelles to make room for increasing amounts of hemoglobin. Hemoglobin is an intracellular metalloprotein used by the body to transport oxygen to the tissues and to some extent carbon dioxide, back to the site of oxygen exchange in the respiratory tract. Hemoglobin's centrally located heme group, which consists of an iron ($Fe^{2+}$) atom covalently bonded to a porphyrin ring, reads spectrophotometrically at a wavelength of 540 nm. The absorbance at this wavelength provides the basis of the assay endpoint.

Particulates, such as nanomaterials, interact with the erythrocyte and exert their cytotoxic effect via a multitude of hypothesized mechanisms dependent upon a variety of characteristics influencing surface reactivity including size, composition, and zeta potential (Nolan et al. 1981; Mayer et al. 2009). First, the direct mechanical interaction between the particulates and the membrane can cause rupture releasing intracellular hemoglobin into the ambient solution. Second, several nanoparticles may act on the membrane of the erythrocyte indirectly via actively oxidizing membrane components and thus increase membrane permeability (Li et al. 2008). Third, altering the ionic composition of the suspension medium through nanoparticle weathering will increase the amount of hemoglobin in the assay medium.

While the hemolytic assay provides rapid and cost-effective means of screening a multitude of nanoparticles, intra-assay variation creates an obstacle when comparing hemolytic assay results within the nanotoxicology community (Dobrovolskaia et al. 2008). Literature suggests that intraspecies variation exists when comparing the susceptibility of the erythrocyte to lysis, thus complicating the hemolytic model. Other factors that may influence intra-assay variation are concentration of erythrocytes used (2%–4%), time from harvest to assay, and time of incubation of erythrocyte with nanoparticles of interest. Interestingly, with the testing of nanoparticles such as colloidal gold (absorbance □ = 540 nm), it must be ensured that the nanoparticles do not interfere with the absorbance of heme. Additional assay interference may stem from the rare occurrence of heme absorbance onto the surface of the nanoparticles. This phenomenon will lead to reduced hemoglobin in the supernatant as the adsorbed hemoglobin will precipitate out at high speed centrifugation. Due to this nanoparticle dependent absorbance, it is likely that the hemolytic assay will need to be adapted to each nanoparticle with different physicochemical properties.

### 30.4.3 Dichlorofluorescin Probe

One central hypothesis in nanotoxicology is that nanoparticles are able to induce cellular dysfunction according to an oxidative stress paradigm. This oxidative stress occurs when intracellular levels of reactive oxygen species (ROS) ($O_2^-$, $HO_2$, $H_2O_2$, and HO) overcome the capability of the cellular defense mechanisms (antioxidants and enzymes) to adequately respond. The 2′,7′-dichloroflourescin diacetate (DCFH-DA) assay has been used repeatedly as an estimation of reactive oxygen species (ROS) production (Wang and Joseph 1999; Lin et al. 2006). In theory, DCFH-DA passively diffuses through the cell membrane. Once inside the cell, DCFH-DA is hydrolyzed by intracellular enzymes to the non-florescent compound DCFH. Intracellular ROS then oxidize the non-fluorescent DCFH to florescent DCF, which may then be read on a conventional plate reader.

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Adapting this assay for use with non-florescent nanoparticles can provide a quick, cost-effective means of screening many nanoparticles for estimation of ROS production.

## 30.5 Biological Response In Vitro and In Vivo

Nanoparticles are known to interact with living organisms down to the cellular level. They have been visualized in in vitro cell culture systems as well as in an in vivo model. Furthermore, both carbonaceous and colloidal nanoparticles have been shown to translocate various biological membrane barriers (epithelium, endothelium, or mucosa) and may eventually enter the bloodstream (Oberdorster et al. 2004; Larese et al. 2009). Once in the bloodstream, nanoparticles (due to their size and surface charge) may enter cross barriers (i.e., blood brain barrier) normally impermeable to other molecules. This increased permeability combined with cell specificity has allowed researchers the enhanced ability to target malignancies in difficult to treat areas (i.e., brain) with increased efficiency (Brigger et al. 2002).

### 30.5.1 Cellular Response to Oxidative Stress

The presence of excessive ROS can cause cellular oxidative stress, which may lead to subcellular damage such as DNA, RNA, protein, mitochondrial, and membrane or other lipid degradation. One of the more commonly probed oxidative stress indictors is protein carbonyl production. The oxidation of protein is damaging to polypeptides and amino acids present in cells and tissues. Protein Carbonyl Content (PCC) and 3-Nitrotyrosine are examples of assays that measure oxidative protein damage. Other techniques used to evaluate the oxidant versus antioxidant biomarkers in vitro are enzyme-linked immunosorbant assays (ELISA), ion exchange chromatography, immunoblotting, and electron paramagnetic resonance.

Some oxidative stress mechanisms are known that describe how pro-oxidants cause protein damage. Alterations in protein function can occur after the formation of covalent bonds between electrophiles and nucleophilic amino acids, the oxidation of nucleophilic amino acids, or the production of reactive nucleophiles.

### 30.5.2 Inflammatory Response

Studies have shown that exposure to nanoparticles to in vitro and in vivo systems may cause the production of inflammatory biomarkers (Warheit et al. 2004; Gurr et al. 2005; Sayes et al. 2006; Zhu et al. 2006). After nanoparticles are internalized by cell, such as phagocytes, the inflammatory cascade may be triggered. Inflammation is the complex biological response of cells and tissues to harmful pathogens and other toxicants. The inflammatory cascade is both a proactive mechanism to remove harmful pathogens and to initiate the production of repair enzymes. Unchecked inflammation can lead to a host of diseases, such as asthma, atherosclerosis, and rheumatoid arthritis; therefore, it is normally tightly regulated by the body.

### 30.5.3 Cell Damage along a Genotoxic Pathway

Nanoparticles have been previously shown to exhibit damage to DNA through DNA oxidation, causing DNA strand breaks as well as binding to DNA at the molecular level (Tsoli et al. 2005). Currently, most nanotoxicology researchers hypothesize that nanoparticles have the potential to induce cell damage in two distinct pathways. First, the small-size and narrow-size distribution of nano-sized particulate matter could lead to stronger interactions with proteins causing structural changes or loss of function due to that fact that particles at the nano scale are about the same size as indigenous molecules within the cell. Because both nanoparticles and molecules, such as proteins, have similar sizes, some researchers believe that they may bind together resulting is disrupted normal cellular function. The second potential toxic pathway is due to the leaching of ions from the surface of a nanoparticle to the surrounding

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cellular matrix. These ions can accumulate in tissues causing, for example, metal poisoning or even cell death. After endocytosis, some nanoparticles disrupt throughout the cell and preferentially deposit on the nuclear or mitochondrial membrane. While it is unclear whether particles translocate through these membrane, damage to both mitochondrial and nuclear DNA has been found (Unfried et al. 2007; AshaRani et al. 2009).

### 30.5.4 Cellular Uptake

Nanoparticles are novel tools in biological and biomedical applications; however, some inherent risks are associated with their incorporation into consumer and medicinal products. An interaction of engineered nanoparticles occurs on a size scale previously seen with the entrance of viral pathogens into a cellular system. Similarly, the uptake of nanoparticles provide a unique way of accessing intracellular compartments albeit intentionally (i.e., medicinally) or unintentionally (i.e., occupationally). In both situations, nanoparticles are capable of intracellular localization through a variety of endocytic mechanisms ranging from the receptor-mediated clathrin mediated endocytosis (CME) to nonspecific macropinocytosis to simple passive diffusion (Qaddoumi et al. 2003; Geiser et al. 2005; Rothen-Rutishauser et al. 2006; Harush-Frenkel et al. 2007a,b; Jiang et al. 2008). Many physicochemical characteristics such as nanoparticle size, degree and type of surface coating, and surface charge are factors influencing nanoparticle endocytosis.

Size is an important characteristic in nanoparticle endocytosis. Many endocytic mechanisms, such as CME and caveolin-mediated endocytosis are structurally size-limited by the protein scaffolding lining the vesicle budding from the membrane surface. Furthermore, a variety of thermodynamic limitations exist that pertain to membrane folding. Calculations based on thermodynamic limitations and cell–ligand interactions have previously calculated that the most efficient receptor-mediated nanoparticle endocytosis occurs at ~30 nm for spherical particles (Freund and Lin 2004; Gao et al. 2005). These confirmations are further strengthened by observations of the uptake efficiency of herception conjugated gold nanoparticles peaking at particle diameters of 25–50 nm (Jiang et al. 2008). While primary particle size is important, nanoparticles may also agglomerate to the micrometer scale. In these instances, these agglomerates may be treated as larger particles. For example, phagocytosis by immune-modulators (macrophages) is responsible for the clearance of micrometer-sized particulates leading to the view that these innate defenses may readily clear micrometer-sized agglomerates of nanoparticles (Oberdorster et al. 2005; Kemp et al. 2008). While size and agglomeration state play a primary role in directing the specific route in nanoparticle uptake, they are not the only factors taken into play.

Surface charge also plays a role in the interactions found between nanoparticles and cell membranes (Harush-Frenkel et al. 2007b; Zhang 2009). For example, negatively charged nanoparticle surfaces, such as particles coated with hydrolipic acids, and positively charged nanoparticles, such as particles coated with polymers, are more capable of incorporation into human cells than particles with little to no surface charge. Furthermore, the charge of a nanoparticle will also dictate the pathway through which the nanoparticle is internalized (Harush-Frenkel et al. 2007b). For example, while uptake of both positively and negatively charged particles are energy and F-actin polymerization dependent, they have been shown to undergo different pathways including macropinocytosis and CME (for the former) and possible dynamin-independent mechanisms (for the latter) (Dausend et al. 2008). While associations between charge and mechanisms of cellular internalization have been made, further research into this topic in needed. In addition to nanoparticle charge, a modified surface coating also influences the uptake by cells.

Another factor dictating nanoparticle cellular uptake and potential toxicity is the adsorption of proteins onto the surface of the nanoparticles. For example, proteins have been shown to coat particles when placed in cell culture media, serum, and bronchiolar lavage fluid (Chen et al. 2005b, 2006; Huang et al. 2007; Lundqvist et al. 2008; Schellenberger et al. 2008). This degree of protein coating or functionalization is dependent upon psychochemical factors such as size and shape. For example, antibody conjugation onto the surface of a nanoparticle increases linearly with respect to the radius (Jiang et al. 2008).

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This association between nanoparticle size and degree of protein binging is due to the high rate of curvature exhibited by the surface of a small nanoparticle that sterically hinders the binding of additional antibodies (Ghitescu and Bendayan 1990). Protein adsorption may be dramatically altered as any single physiochemical change is made. It is this aforementioned protein adsorption theory that necessitates the importance of proper characterization of nanomaterials as well as puts forth the complicating task of linking nanomaterial physicochemical properties with their biological endpoint.

## 30.6 Standardization of Methods

Along with the growing amount of nano-focused environmental health and safety researchers, published literature, organized meetings and conferences, and student training programs, the nanotoxicology community is making substantial progress toward standardizing methods and techniques used in this nascent scientific discipline. Experts from fields of toxicology, medicine, chemistry, biology, physics, and engineering study issues including hazard identification, exposure science, risk assessment and management, and public health and policy on a daily basis. As the field moves forward in making critical decisions in standardizing laboratory practices and operating procedures, it is critical that novel techniques and nontraditional use of techniques continue to be researched and published in the peer-reviewed literature.

As commented in the 2008 *Nanotoxicology 2nd International Conference* proceedings by Krug (2009), it is essential to compare the experimental designs and resultant data sets that are produced by the people working in nanotoxicological laboratories. At that time, there is an overwhelming criticism to the nanotoxicology community regarding the lack of methods standardization and the differing results that have been published. Most researchers agree, however, that the inherent nature of nanoparticles gives rise to contradictory nanotoxicity reports. Some of the physicochemical parameters that lead to the so-called discrepancies include nanoparticle synthesis/production conditions, the use, misuse, or absence of colloidal stabilizers in particle suspensions, dosimetric and concentration measurements, exposure time, and interference of traditional colorimetric probes with particle spectroscopic features. Many working groups and subsequent review manuscripts have identified the following critical needs for the future of nanotoxicology research:

- Toxicokinetic and toxicodynamic profiles of nanoparticles in in vivo systems
- Identification of both toxic and nontoxic cellular responses for nanoparticles along specific pathways
- Additional exposure evaluation research
- Refinement of existing and development of new analytical tools for identifying and measuring nanoparticles in complex matrices
- Direct and indirect effects of nanoparticles on the immune system and genotoxicity
- Development of predictive models and open access databases of nanotoxicity results

Without chemical and physical characterizations of the materials and their surrounding matrix, research on their toxicological effects may not be useful because it would be difficult to make substantial conclusions or draw comparisons between the relationship between specific particle types and their mode of action in biological systems. Furthermore, development of risk assessment models is hindered until such standardizations are in place.

### 30.6.1 Physicochemical Characterization Data in the Nanotoxicology Literature

The properties of nanomaterials are predominantly associated with their nanometer-scale size and structure, size and structure-dependent electronic configurations, and an extremely large surface area-to-volume ratio relative to larger-sized chemicals and materials. The main characteristic of nanomaterials

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is their size, which falls in the transitional zone between individual atoms or molecules and the corresponding bulk materials (Nel et al. 2006). Particle size and surface area are important material characteristics from a toxicological and health perspective because as the size of a particle decreases, its surface area increases, which allows a greater proportion of its atoms or molecules to be displayed on its surface rather than within the interior of the material. These atoms or molecules on the surface of the nanomaterial may be chemically and biologically reactive, potentially contributing to the development of adverse health effects. Other physical and chemical properties such as shape, surface coating, aggregation potential, and solubility may also affect the physicochemical and transport properties of the nanomaterial with the possibility of negating or amplifying any associated size-related effects.

### 30.6.2 Conclusions

The field of nanotoxicology is growing, but has its roots in the toxicology of ultrafine particles. Nanoparticles will require careful evaluation with reference to routes of exposure, dosemetrics, biocompatibility, and toxicokinetics/toxicodynamics. The inherent size scale of nanomaterials links the immune systems as an obvious target. Nonetheless, the physicochemical and biological rules by which adverse events will be determined are poorly understood. Going forward, the enterprise will continue to require cross-collaboration between physical and biological scientists and highly integrated training of the next generation of scientists and engineers.

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