7 Preclinical Characterization of Engineered Nanoparticles Intended for Cancer Therapeutics

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7.1 INTRODUCTION

As discussed in other chapters of this book, nanotechnology offers the research community the potential to significantly transform cancer diagnostics and therapeutics. Our ability to manipulate the biological and physicochemical properties of nanomaterial allows for more efficient drug targeting and delivery, resulting in greater potency and specificity, and decreased adverse side effects. The combinatorial possibilities of these multifunctional platforms have been the focus of considerable research and funding, but realization of their use in clinical trials is highly dependent on rigorous preclinical characterization to meet regulatory provisions and elucidated structure–activity relationships (SARs). A rational characterization strategy for biomedical nanoparticles contains three essential components (see Figure 7.1): physicochemical characterization, in vitro assays, and in vivo studies.

7.1.1 Physicochemical Characterization

One of the major criticisms of early biomedical nanotechnology research was the general lack of physicochemical characterization that did not allow for the meaningful interpretation of resulting data or inter-laboratory comparisons. Traditional small molecule drugs are characterized by data that contribute to the chemistry, manufacturing and controls (CMC) section of the investigational new drug (IND) application with Food and Drug Administration (FDA), which include their molecular weight, chemical composition, identity, purity, solubility, and stability. The instrumentation to ascertain these properties has been well established, and the techniques are standardized. Engineered nanomaterials have dimensions between small molecules and bulk materials and often exhibit different physical and chemical properties than their counterparts. These physical and chemical

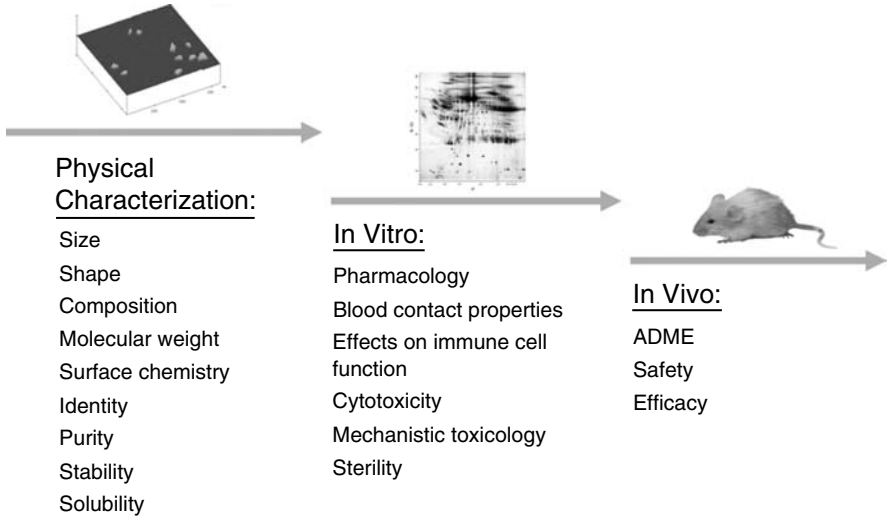


FIGURE 7.1 An assay cascade for preclinical characterization of nanomaterials.

properties influence the biological activity of nanoparticles and may depend on parameters such as particle size, size distribution, surface area, surface charge, surface functionality, shape, and aggregation state. Additionally, because most nanoparticle concepts are multifunctional, the distribution of targeting, imaging, and therapeutic components can also have dramatic effects on nanoparticle biological activity. There is a need to establish and standardize techniques to define these nanoparticle attributes.

There is now ample evidence that size and surface characteristics can dramatically affect nanoparticle behavior in biological systems.[1–4] For instance, a decrease in particle size leads to an exponential increase in surface area per unit mass, and an attendant increase in the availability of reactive groups on the surface. Nanoparticles with cationic surface character have a notably increased ability to cross the blood–brain barrier compared to nanoparticles with anionic surfaces.[5] In general, surface area, rather than mass, provides a better fit of dose–response relationships in toxicity studies for particles of various sizes.[6,7] Physicochemical characterization of properties, such as size, surface area, surface chemistry, and aggregation/agglomeration state, can provide the basis for better understanding of SARs.

7.1.2 In Vitro Characterization

In vitro assays enable the isolation and analysis of specific biological and mechanistic pathways under controlled conditions. While many in vitro assays for nanomaterials will be similar to those used for traditional drugs, others will address mechanisms more specific to nanoparticles, such as oxidative stress. Noncellular assays measuring processes, such as protein adsorption, will also be an important accompaniment to cell-based assays. For example, monitoring the profile of serum proteins that absorb to nanoparticles in an in vitro environment may further our understanding of how nanoparticles interact with components of the reticuloendothelial system (RES) in vivo.[8,9] Additionally, proteomics and toxicogenomics can be employed to potentially identify biomarkers of toxicity related to nanomaterial exposure.[10]

7.1.3 In Vivo Characterization

In vivo studies must be conducted to better understand the safety and behavior of nanoparticles in a living organism. As with any new chemical entity (NCE), the nanoparticle formulations' pharmacological and toxicological properties (i.e., ADME/Tox) need to be thoroughly characterized. In vivo studies should include examination of nanoparticles' effects on various organs and systems, such as the liver, heart, kidney, and immune system.

In this chapter, we outline the scientific rationale underlying the development of an assay cascade, with special attention paid to the selection and adaptations of assays and analytical protocols needed to extract meaningful efficacy and safety data from nanomaterials. These are presented in the following four sections: (1) physicochemical characterization, (2) in vitro pharmacology and toxicology assessment, (3) in vivo pharmacology and toxicology assessment, and (4) immunotoxicity. Standardized characterization of nanomaterials will facilitate better inter-laboratory comparison of results and will enhance the quality of scientific data submitted by investigators in support of their IND applications.

7.2 PHYSICOCHEMICAL CHARACTERIZATION

The physicochemical characteristics of nanomaterials can affect their cellular uptake, binding to blood proteins, access to target sites, and ability to cause damage to cells and tissue.[11] Standard methods for physicochemical characterization of nanomaterials will provide the basis for rational product development as well as consistent and interpretable results for tests of efficacy and safety. Few examples of standard characterization criteria exist in the literature, and there is as yet no

consensus as to what measurement criteria are appropriate for any given nanomaterial product. However, it is clear that the diversity and complexity of nanomaterials used in biomedical applications dictates a more comprehensive and strategic approach to characterization than has been applied to date.

There are many varieties of nanomaterials currently being investigated for biomedical applications, especially for cancer diagnosis, imaging, and targeted drug delivery. These nanomaterials may be classified under several broad categories:

- Organic nanoparticles (e.g., dendrimers, polymers, functionalized fullerenes)
- Inorganic nanoparticles and organic–inorganic hybrids (e.g., iron oxide core particles, quantum dots)
- Liposomes and other biological nanomaterials

Each category of nanomaterial has a distinctly different composition that gives rise to different physical properties, such as solubility, stability, surface characteristics, and functional capabilities. Even within a single category, there can be a tremendous variety of product compositions, each with unique physical and chemical characteristics, and each requiring a different strategy for measuring those characteristics. This section examines the various categories of nanoparticles, and the tools and instrumentation available to address physicochemical characterization.

7.2.1 Physicochemical Characterization Strategies

Successful characterization strategies will enable one to begin associating the physicochemical properties of a nanomaterial with its in vivo behavior (i.e., SARs). This is an important step in the development of any material used for medical applications. For small molecules, the basis of most traditional drugs, the characterization techniques have been well established and standardized to determine their attributes, such as melting point, boiling point, molecular weight and structure, identity, composition, solubility, purity, and stability. These characteristics are measured and adequately defined using elemental analysis, mass spectrometry (MS), nuclear magnetic resonance (NMR), ultraviolet-visible (UV–vis) spectrophotometry, infrared (IR) spectroscopy, high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), polarimetry, and other common analytical methods. Each of these individual techniques provides unique information about the sample, while together they provide the foundation for product quality control, manufacturing, and regulatory approval.

Many of the techniques used to characterize small molecules apply to nanomaterials. However, due to the composite nature of nanomaterials, the definition and measurement of these attributes can be quite different. To fully understand the attributes of a nanomaterial, additional characterizations are needed, such as size, surface chemistry, surface area, polydispersity, and zeta potential (see Figure 7.2). A comprehensive analysis of these properties is necessary to better understand in vivo effects and to allow for greater consistency and reproducibility in their preparation. The requirements set by regulatory bodies for quality control and consistency of biomedical nanomaterials are likely to be as stringent as those for small molecule preparations, but the path to verifying quality will require a more sophisticated approach. At the core of this analysis is an array of tools and instrumentation that are particularly well suited to measuring the properties of nanomaterials.

7.2.2 Instrumentation

7.2.2.1 Spectroscopy

Many traditional analytical methods can be applied to the characterization of nanomaterials. For example, NMR is extensively used to characterize dendrimers, polymers, and fullerenes derivatives, and provides unique information on the structure, purity, and functionality.[12–14]

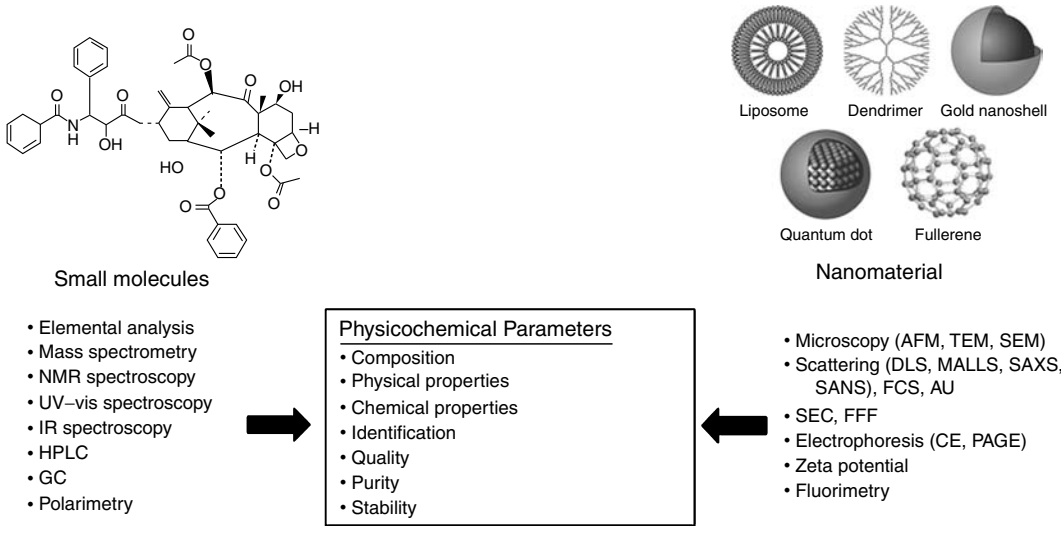


FIGURE 7.2 Physicochemical characterization methods and instrumentation for small molecules and nanotechnology.

In addition, the average number of terminal capping groups, number of small molecule ligands, and drugs in a multifunctional nanomaterial can be ascertained by comparing the integration values with chemical shifts unique to the ligands. UV–vis absorption spectrophotometry is also extensively used to identify and quantify the chromophore present in the preparation by using its extinction coefficient. Spectrofluorimetry is used in cases where the material has inherent fluorescence (such as quantum dots) or labeled with a fluorescence probe. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS is used extensively for macromolecules, dendrimers, and polymers to determine the molecular weight and utilize novel matrices to minimize the fragmentation of the macromolecule before reaching the detector. In the case of lower generation dendrimers, the presence of impurities, incomplete reaction, and reaction byproducts can be easily determined using MS.

7.2.2.2 Chromatography

Liquid chromatography methods such as analytical HPLC and size-exclusion chromatography (SEC; also called gel-permeation chromatography or GPC) utilize a column to separate components of a mixture in a liquid mobile phase based on their interaction with a solid stationary phase. The eluents are passed through UV–vis and fluorescence detectors with a flow cell where the absorbance and fluorescence is recorded to determine the purity of the sample. Although these techniques are suitable for stable polymers, dendrimers,[15] functionalized fullerenes, and protein- and peptide-based nanomaterial, they are not suitable for particles that degrade under experimental conditions or have excessive nonspecific binding to the solid matrix.

7.2.2.3 Microscopy

Scanning probe microscopy (SPM) techniques can be employed to measure the size, topography, composition, and structural properties of nanoparticles. Related techniques such as scanning tunneling microscopy (STM), electric field gradient microscopy (EFM), scanning thermal microscopy, and magnetic field microscopy (MFM) combined with atomic force microscopy (AFM), can be used to investigate the structural, electronic, thermal, and magnetic properties of

a nanomaterial. AFM uses a nanoscale probe to detect the inter-atomic forces and interactions between the probe and the material being analyzed and is capable of determining size and shape within a spatial resolution of a few angstroms.[16] Apart from the ability to measure the particle size in a dry state as well as in aqueous and physiological conditions, AFM is a useful tool to probe the interaction of nanoparticles with supported lipid bilayers. This technique has been successfully used to compare nanoparticle interactions in in vitro cell assays.[17,18] The ability to image under physiological conditions makes AFM a powerful tool for the characterization of nanoparticles in a dynamic, biological context. A variant of this method, molecular recognition force microscopy (MRFM), can be employed to study the specific ligand–receptor interactions between nanoparticles and their biological targets.

Optical microscopy techniques are useful at the micron scale and are extensively used for imaging structural features. Fluorescence and confocal microscopy may be used to determine cellular binding and internalization of fluorescent-labeled nanoparticles[19] or those that are inherently fluorescent, such as quantum dots. But a more precise analysis of nanomaterial size and other direct measurements of physical properties will require a more sophisticated and specialized set of microscopic and spectroscopic techniques.

Scanning electron microscopy (SEM) provides information on the size, size distribution, shape, and density of nanomaterials. Transmission electron microscopy (TEM) and high-resolution TEM are more powerful than SEM in providing details at the atomic scale and can yield information regarding the crystal structure, quality, and grain size. TEM can be coupled with other characterization tools, such as electron energy loss spectrometry (EELS) or energy dispersive x-ray spectrometry (EDS), to provide additional information on the electronic structure and elemental composition of nanomaterials. Samples for TEM are evaluated dry or in a frozen state, under high-vacuum conditions. Nanoparticles analyzed by this instrument must therefore be stable under these extreme conditions. Additionally, while considered a gold standard of microscopic characterization methods, TEM requires a great deal of skill and time to obtain good data. In principle, when establishing characterization protocols, TEM can be used to validate characterization methods that are easier to use on a routine basis. Further description of analytical technologies as they apply to the measurement of specific nanomaterial properties is provided in the following sections.

7.2.2.4 Size and Size Distribution

Size is one of the critical parameters that dictate the absorption, biodistribution, and route of elimination for biomedical nanomaterials.[20] Generally, nanoparticles with dimensions of less than 5–10 nm are rapidly cleared after systemic administration, while particles from 10 to 70 nm in diameter may penetrate capillary walls throughout the body.[21,22] Larger particles 70–200 nm often remain in circulation for extended times.[22,23] This general correlation of biodistribution and elimination with respect to size may vary greatly depending on nanoparticle surface characteristics.

Specifically in cancer applications, size is an important factor in the accumulation of therapeutic nanomaterials in tumors, usually as a result of enhanced permeation and retention (EPR), caused by local defects in the vasculature and poor lymphatic drainage.[24] Particle size can be precisely tuned to take advantage of this phenomenon and passively target and deliver a therapeutic payload to tumors.[25–27]

Depending on the category of the nanomaterial, synthesis and scale-up can be problematic. Most biomedical nanomaterials for therapeutic and diagnostic applications are complex and involve some combination of molecular self-assembly, encapsulation, *and*/or the use of *nano*-sized metal or polymer cores, surfactants and/or proteins to impart solubility and functionality. Due to inherent variability in the manufacturing process, one rarely achieves a monodisperse, homogeneous product. It is therefore important to ascertain the precise size, size distribution, and polydispersity index (PDI) of the material. There are several techniques available to assess these parameters, including electron microscopy, AFM, and light scattering. Light scattering

techniques can measure overall size and polydispersity of the particles. TEM is powerful in ascertaining the homogeneity of nanoparticles with encapsulated metals and in determining core size. With knowledge of nanoparticle geometry and size, surface area can also be estimated.

For biological applications, it is important to measure the physical characteristics of the nanomaterial in isotonic solution at physiological pH and temperature. The hydrodynamic size can be measured under these conditions using dynamic light scattering (DLS) (also known as photon correlation spectroscopy [PCS] and quasi elastic light scattering [QELS]) and analytical ultracentrifugation (AU). In a DLS experiment, the effects of Brownian motion (particle movement caused by random collisions in solution) provide information on particle size and size distribution. The sample is illuminated with a laser, and the intensity fluctuations in the scattered light are analyzed and related to the size of the suspended particles. This technique is useful in determining whether the nanomaterial is monodisperse in size distribution. These data are influenced by the viscosity and the temperature of the medium, since Brownian motion depends on these factors. The pH of the medium and salt concentration may also affect the degree of agglomeration in some samples. With DLS, sample preparation is easy, the measurement is quick, and data are reproducible on larger sample volumes compared to microscopy techniques; however, better standardization of procedures, conditions, and data analysis tools will be required. Static light scattering provides information on molar mass and root-mean-squared (rms) radius for fractionated or monodisperse samples. One limitation of light scattering instruments is the inability to measure the size when the nanoparticles absorbs in the wavelength of the laser being used. Small-angle x-ray scattering (SAXS)[28] and small-angle neutron scattering (SANS)[29] can be used to measure the size, shape and orientation of components. Due to their cost and infrastructure requirements, there is limited availability of these instruments. For fluorescent nanomaterials such as quantum dots, size can be measured using fluorescence correlation spectroscopy (FCS).[30]

The hydrodynamic size of nanoparticles can also be measured with AU, which is traditionally used to measure the size of proteins.[31] The instrument spins the protein sample solution under high vacuum at a controlled speed and temperature while recording concentration distribution at set times. Even though this technique is designed to measure the size of proteins in solution, it has potential applications in the measurement of the hydrodynamic size of nanoparticles samples that are stable under the experimental conditions. Fractionation using SEC separates stable polymers into individual components and helps in the determination of the PDI. In the case of unfractionated samples, batch mode measurement provides averaged quantities such as weight-averaged molar mass and *z*-average rms radius. This technique is especially useful when combined with a refractive index detector to obtain absolute molecular weight for very high molecular weight polymers where traditional MS methods fail.

In cases where the separation and fractionation of nanomaterial is not possible using a column with a stationary phase, such as when the nanomaterial may interact with the column packing material and render it unstable, asymmetric-flow field flow fractionation (AFFF) is useful.[32] In AFFF, separation occurs when the sample passes through a narrow channel with a cross-flow through a porous semi-permeable membrane. The faster moving smaller particles rise to the top of the flow and come out first followed by larger particles that stay closer to the membrane and migrate more slowly. One advantage in this method is that there is no stationary phase in the separation: the sample injected comes out intact with little loss of material due to nonspecific binding. This feature is particularly useful for less stable nanoparticles such as liposomes, or for polymer- or protein-coated metal nanoparticles that would otherwise interfere with the performance of a traditional GPC column. The efficiency of separation for AFFF is not as good as with GPC, but there have been recent improvements in instrumentation that are closing the gap in performance. For both GPC and AFFF, the quantity and hydrodynamic size of the nanoparticles are detected in eluted peaks by measuring absorbance, refractive index, and light scattering.

In addition to size, the shape of a nanoparticle may affect its distribution and absorption in the body. Spherical, tubular, plate-like, or nano-porous materials of the same composition can vary significantly in their surface energy, biological activity, and access to different physiological structures, such as cell walls, capillary vessels, etc. Methods such as AFM, SEM, TEM, and STM can be used to determine the distribution of shape in a nanoparticle preparation.

7.2.2.5 Surface Characteristics

Surface characteristics contribute to the nanoparticle's solubility, aggregation tendency, ability to traverse biological barriers (such as a cell wall), biocompatibility, and targeting ability. The nanoparticle surface is also responsible for interaction and binding with plasma proteins in vivo, which in turn may alter the nanoparticle's distribution and pharmacokinetics. For multifunctional nanoparticles, modifying agents are often attached to the surface to bind to receptors in target tissues and organs. The presence of charged functionalities on the nanoparticle surface may increase nonspecific uptake, making the preparation less effective in targeting. It has been shown that dendrimer nanoparticles displaying positively charged amine groups on their surface can be significantly more hemolytic and cytotoxic than nanoparticles displaying negatively charged carboxylates.[20] The negatively charged nanoparticles were also cleared more slowly from the blood compared to positively charged species, following intravenous administration to rats.[20] Another potential effect of surface charge is to alter a nanoparticle's ability to penetrate the blood–brain barrier. Studies have shown that for emulsifying wax nanoparticles, anionic surfaces were superior to neutral or cationic surfaces for penetration of the blood–brain barrier.[33]

Surface characteristics can be tuned to improve receptor binding, reduce toxicity, or alter biodistribution. For example, when the above-mentioned dendrimers were acetylated to neutralize exposed surface charges, the toxic effects of the nanoparticles were also neutralized.[20,34] Surface properties can also lead to toxicity through interaction with molecular oxygen, leading to oxidative stress and inflammation. Electron capture at the surface of the nanoparticle results in the formation of the superoxide radical, which can set off a cascade of reactions (e.g., through Fenton reaction or disumation) to generate reactive oxygen species (ROS). ROS generation has been studied extensively for inhaled nanoparticles,[11,35] and has been observed in engineered nanoparticles such as fullerenes, single walled nanotubes (SWNTs), and quantum dots.[6,36–44] Studies have shown a direct correlation between nanoparticle surface area and ROS-generating capacity and inflammatory effects.[11]

The nature and integrity of nanomaterial surfaces must be established through analytical measurements to ensure product quality and account for surface-dependent effects on biodistribution and toxicity. Potentiometric titrations provide crucial information on the net charge of a nanoparticle, and include zeta potential analysis, which provides information on the net charge and distribution under physiological conditions. Polyacrylamide gel electrophoresis (PAGE) analysis of dendrimers and other nanopolymers yields information on the molecular weight and the polydispersity of nanoparticles (such as trailing generations in dendrimer populations) based on their migration through the gel under an electric field. PAGE is also a powerful tool in the qualitative analysis of bioconjugates of nanomaterials with DNA, oligonucleotides, antibodies, and other ligands. Further analysis of the surface charge distribution and polydispersity of nanomaterials can be conducted using CE. MS is also effective in ascertaining the number and distribution of charges, especially for smaller and purer nanoparticles with known molecular weight.

7.2.2.6 Functionality

Analysis of the functional components of nanomaterials, such as targeting, imaging, and therapeutic agents, is critical to understand the in vivo efficacy of the preparation. Characteristic

features of functional components include their quantity, distribution, orientation, and activity. For targeting agents, a key advantage of their use in nanoparticles is their ability to provide increased avidity to the target due to polyvalency. The level of polyvalency and activity of targeting agents can be monitored using surface plasmon resonance (SPR) to measure the rate constants for nanoparticle association and dissociation. During preclinical development, the affinity of nanomaterial preparations for their target molecule/receptor can be analyzed using SPR and compared to data obtained for binding to cellular receptors in culture.[45]

The average number of targeting agents per nanoparticle has to be optimized for both solubility and binding affinity. Affinity chromatography or SEC can be employed with some nanoparticles to separate nanoparticles with targeting agents from those without targeting agents. In nanoparticles containing antibodies[19,46] or proteins, quantification can be achieved using an enzyme-linked immunosorbent assay (ELISA) or bicinchoninic acid assay (BCA) if the inherent property of the nanoparticle itself does not interfere with the assay. In the case of dendrimers, NMR has been successfully applied to analyze the average number of targeting agents by comparing the integration values of the signals associated with the targeting agents to those belonging to the dendrimer. This is still an averaged technique that cannot distinguish the distribution of targeting agent density on a population of nanoparticles.

For targeted drug delivery applications, it is obviously important for both the targeting and therapeutic agents to be on the same particle. If the therapeutic has UV–vis absorption, it can be quantified using UV–vis spectroscopy with the extinction coefficient of the drug. HPLC analysis is possible in some cases to evaluate the amount of the drug present in a known amount of material, after isolating the drug from the sample.

7.2.2.7 Composition and Purity

Biomedical nanomaterials can be comprised of a wide variety of substances, including polymers, metals and metal oxides, lipids and other organic compounds, and large biomolecules such as protein or DNA. In most cases, the nanomaterials combine two or more of these substances, such as in a core or shell of a particle, and in encapsulated or conjugated material. Analysis of chemical composition will be critical for confirming the purity and homogeneity of nanomaterial product preparations.

Elemental analysis, such as CHN analysis, is most often used to ascertain the purity of small molecules. For nanomaterials, elemental analysis can be used to determine the composition and ratios of different elements present in the sample. For example, this technique can be used to determine the amount of linker present, if a unique element (such as sulfur) has been employed in the synthesis. In the case of core–shell metal nanoparticles, the ratio of core to shell material ratios can be determined.

Atomic absorption (AA) and atomic emission (AE) spectroscopies can also be utilized to determine the composition of nanomaterials. For imaging applications using iron oxide nanoparticles or gadolinium (Gd)-based chelates, composition analysis is very important to quantify metals present in the preparation which influence imaging efficacy. Inductively coupled plasmon optical emission spectroscopy (ICP-OES) is very sensitive to determine the amount of Gd in such contrast agent conjugates.[47] Specific T1/T2 relaxivities of magnetic resonance contrast agents can, of course, be assessed under in vitro conditions in the actual MRI instrument.

The purity of synthetic small molecules can be determined with a high degree of certainty since the analyte usually consists of a single component. With nanomaterials, purity must be determined in the context of multiple layered, conjugated, and encapsulated components. Purity analysis must account for the presence of solvents, free metals and chelates, unconjugated therapeutic or other agents, precursors, dimers, etc., that result in artifacts and side products of the preparation.[48] Characterization of the inhomogeneity in ligand distribution is very important for efficacy as well as testing batch-to-batch reproducibility.[49] Proper methods and techniques to detect the

presence of all these entities are required to ensure the purity and quality of nanomaterial preparations and to further expand our understanding of SARs.

7.2.2.8 Stability

The ability of multifunctional nanoparticles to combine targeting, therapeutic, and imaging modalities is a key aspect of their versatility and anticipated clinical impact.[50–52] With such complex compositions, the stability of all the components in nanoparticles is essential to their biological function. Premature release of any of the components from the composite preparation may render it ineffective. For example, in a nanodelivery system containing a targeting agent and a drug, the nanoparticles with the drug cannot bind to the desired targeting site if the targeting agent is prematurely cleaved or released. If the drug is prematurely released, even if the nanoparticle reaches its target, there will no longer be a therapeutic benefit.[53] For this reason, it is important to determine the in vitro functional component stability under physiological conditions.

For a nanomaterial providing targeted or timed-release drug delivery with an encapsulated drug, the release profile should be determined at different ionic strength, pH, and temperature conditions. Examples of such conditions include the stability at pH 7.4, in buffers such as phosphate buffered saline (PBS), and serum at 37°C. There are many nanoparticle designs being pursued which incorporate the selective release of components triggered by an external stimulus after targeted delivery. If a therapeutic attached to a nanoparticle uses a cleavable linkage, the efficiency of release should be determined under the expected cleavage conditions.[54]

In cases where a metal complex is used (for example, a Gd chelate for enhanced MRI contrast), the stability constants for the encapsulation or complexation should be determined, since any release of free heavy metal will increase the in vivo toxicity of the preparation.[55] The potential in vivo application of quantum dots has raised some concerns that the CdSe core might be exposed by the breakdown of its protective polymer or inorganic shell, releasing the highly toxic heavy metal Cd^{2+} ions into the bloodstream.[56] The quantum dot shells have been designed to be protective, but their long-term stability (e.g., susceptibility to Cd leaching) has not been established. Studies conducted on primary hepatocytes in vitro suggest that CdSe core quantum dots may be acutely toxic under certain conditions.[57] Other studies suggest that under physiological conditions, appropriately coated quantum dots do not expose the host organism to toxic levels of the core material.[58–60] Apparently conflicting evidence as to the safety of quantum dots highlights the necessity of clearly and objectively establishing the stability of these nanoparticles under physiological conditions using standardized methodologies.

It is also important to determine the stability of the nanoparticle under nonphysiological conditions to account for the effects of short-term and long-term storage, lyophilization, ultrafiltration, thermal exposure, pH variation, freeze–thawing, and exposure to light.

In summary, adequate physicochemical characterization of nanomaterials should be included as an essential requirement for preclinical characterization. Just as molecular characterization forms the basis of dosing and toxicity studies for small molecule therapeutics and diagnostic compounds, physicochemical characterization provides the foundation for dosing and toxicity studies for nanomaterials intended for clinical applications. Standardized protocols are being established by Standards Developing Organizations, such as the International Standards Organization (ISO) and American Society for Testing and Materials (ASTM), for characterizing the many types of biomedical nanomaterials being developed today for human use. Additionally, standardized reference material (SRM) will enable analytical technologies to be calibrated and protocols to be tested for consistency and to facilitate inter-laboratory comparisons.

To better control for the results of in vivo studies of nanomaterial absorption, distribution, metabolism, elimination, and toxicity, it will be necessary to examine the material in the same physicochemical state as would be found under physiological conditions. Particle-specific attributes that should be evaluated include surface characteristics, chemical composition, shape, size,

and ligand dispersity. Additional properties that are influenced by experimental conditions include solubility, stability, protein binding, and aggregation state. Knowing the exact physiological conditions in different tissues and organs and developing a means to either replicate those conditions or measure physicochemical properties in situ is a significant challenge. But continued studies in this area will provide further data to elucidate the linkages between physicochemical characteristics of nanomaterials and their biological effects (i.e., SARs).

7.3 IN VITRO PHARMACOLOGICAL AND TOXICOLOGICAL ASSESSMENTS

Prior to filing an IND or investigational device exemption (IDE) application with the FDA and subsequent clinical testing in humans, a new product must be adequately studied for efficacy and safety using animal models. The cost- and labor-intensiveness of these in vivo studies impel drug and device researchers to make use of predictive in vitro methodologies wherever technology permits. In vitro models can serve as an initial assessment of a nanomaterial's efficacy and absorption, distribution, metabolism, elimination, and toxicity (ADME/Tox), allowing a more strategic approach to animal studies. Used iteratively with in vivo studies, the two approaches can inform each other and help narrow investigations of the physiological and biochemical pathways that contribute to ADME/Tox behavior.

A variety of cell-based in vitro systems are available, including perfused organs, tissue slices, cell cultures based on a single cell line or combination of cell lines, and primary cell preparations freshly derived from organ and tissue sources. In vitro models allow examination of biochemical mechanisms under controlled conditions, including specific toxicological pathways that may occur in target organs and tissues. Examples of mechanistic toxicological endpoints assessed in vitro include inhibition of protein synthesis and microtubule injury. These mechanistic endpoints can provide information not only as to the potential mechanisms of cell death, but also can identify compounds that may cause chronic toxicities that often results from sublethal mechanisms that may not cause overt toxicity in cytotoxicity assays. Common mechanistic paradigms associated with nanoparticle toxicity include oxidative stress, apoptosis, and mitochondrial dysfunction. Due to the nanoparticle- and approach-specific nature of pharmacology studies, it is beyond the scope of this chapter to discuss pharmacological assay specifics. Where appropriate models exist, chemotherapeutic efficacy can be examined in vitro. In certain cases, targeting of chemotherapeutic agents may be demonstrated as well, using optimized treatment/wash-out schemes in cell lines expressing the targeted receptor. Though nanoparticle metabolism or enzyme induction has yet to be demonstrated, certain nanomaterials with attractive chemistries may be subjected to phase-I/II metabolism and induction studies using cell-based, microsomal, and/or recombinant enzyme systems.

7.3.1 Special Considerations

Many of the standard methods used to evaluate biocompatibility of new molecular and chemical entities are fully applicable to nanoparticles. However, existing test protocols may require further development and laboratory validation before they become available for routine testing. Careful attention must be paid to potential sources of interference with analytical endpoints that may lead to false-positive or false-negative results. Nanoparticle interference could result from: interference with assay spectral measurements; inhibition/enhancement of enzymatic reactions[61,62]; and absorption of reagents to nanoparticle surfaces. In the event of nanoparticle interference, additional sample preparation steps or alternative methods may be required.

When evaluating the results of in vitro assays, it is important to recognize that dose–response relationships will not always follow a classical linear pattern. These atypical dose–response relationships have previously been attributed to shifts between the different mechanisms underlying the measured response.[63] In the case of nanomaterials, it is also important to bear in mind that

concentration-dependent changes in the physical state (e.g., aggregation state, degree of protein binding) may also result in apparent nonlinearity.

Another key consideration when evaluating the results of nanoparticle research is the impact of dose metric (e.g., mass, particle number, surface area), sample preparation (e.g., sonication), and experimental conditions (e.g., exposure to light) on the interpretation of results. For example, surface area or particle number may be a more appropriate metric than mass when comparing data generated for different sized particles. This has been shown to be the case for 20- and 250-nm titanium dioxide nanoparticles, in which lung inflammation in rats, as assessed by percentage of neutrophils in lung lavage fluid, correlated with total surface area rather than mass.[64] The importance of experimental conditions in study design is highlighted by an investigation of functionalized fullerenes, demonstrating that the cytotoxicity of dendritic and malonic acid functionalized fullerenes to human T-lymphocytes in vitro is enhanced by photoexcitation.[41] The standardization of these experimental variables should limit inter-laboratory variability and make data generated more comparable.

7.3.2 In Vitro Target-Organ Toxicity

A recently published report from the International Life Sciences Institute Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group[73] recommends the inclusion of several specific in vitro assays in a standard protocol of safety assessment. Much of the report focused on toxicity screening for environmental exposure to nanoparticles and thus emphasized environmentally relevant exposure routes. However, in addition to the in vitro examination of so-called portal-of-entry tissues, the report expressed the need for inclusion of potential target organs. The liver and kidney were selected as ideal candidates for these initial in vitro target organ toxicity studies, since preliminary investigations (discussed below) have identified these as the primary organs involved in the accumulation, processing, and eventual clearance of nanoparticles.

The liver has been identified in many studies as the primary organ responsible for reticuloendothelial capture of nanoparticles, often due to phagocytosis by Kupffer cells.[65–67] Fluorescein isothiocyanate-labeled polystyrene nanoparticles and radiolabeled dendrimers, for example, are rapidly cleared from the systemic circulation by hepatic uptake following intravenous injection.[4,68] Hepatic uptake has also been shown to be a primary mechanism of hepatic clearance for parenterally administered fullerenes, dendrimers, and quantum dots.[20,69,70] In addition to hepatic accumulation, nanoparticles have also been shown to have a detrimental effect on liver function ex vivo and alter hepatic morphology. Hepatocytes isolated from rats intravenously administered polyalkylcyanoacrylate nanoparticles had diminished secretion of albumin and decreased glucose production.[71] This alteration in albumin synthesis was also observed in freshly isolated rat hepatocytes exposed to polyalkylcyanoacrylate nanoparticles. Dendrimers have also been shown to cause liver injury. Repeated dosing of mice with polyamidoamine (PAMAM) dendrimers resulted in vacuolization of the hepatic parenchyma, suggesting lysosomal dysfunction.[72]

Sprague–Dawley rat hepatic primary cells and human hepatoma Hep-G2, selected for in vitro hepatic target organ toxicity assays, have a long history of use in toxicological evaluation.[73–75] Hep-G2 cells were chosen since they are a readily available hepatocyte cell line with high metabolic activity.[76] Rat hepatic primary cells were also chosen for toxicological studies, since hepatic primary cells in culture are more reflective of in vivo hepatocytes with regard to enzyme expression and specialized functions. One survey found rat hepatic primary cells up to ten times more sensitive to model hepatotoxic agents than established hepatic cell lines.[77] Rat hepatic primaries represent a suitable alternative to human hepatic primary cells, which are scarce, costly, and suffer from interindividual variability.

Preliminary pharmacokinetic studies of parenterally administered, radiolabeled carbon nanotubes, dendritic fullerenes, and low generation dendrimers in rodents have identified urinary

excretion as the principal mechanism of clearance.[78–80] A variety of engineered nanoparticles, including actinomycin D-loaded isobutylcyanoacrylate, doxorubicin-loaded cyanoacrylate, and dendrimer nanoparticles, have also been shown to distribute to renal tissue following parenteral administration in rodents.[68,81,82] In the case of the doxorubicin-loaded cyanoacrylate nanoparticles, doxorubicin renal distribution was increased due to capture of the nanoparticles by glomerular mesangial cells. This resulted in a shift in the primary target organ from the heart to the kidney. Doxorubicin-induced renal injury presented as a severe proteinuria. Kidney injury has been demonstrated for other nanomaterials as well. Nano-zinc particles, for example, caused severe histological alterations in murine kidneys and Q-dots were shown to be cytotoxic to African green monkey kidney cells.[83,84]

The porcine renal proximal tubule cell line, LLC-PK1, was selected as a representative kidney cell line, since it is readily available through ATCC and has been used extensively in nephrotoxicity screening and mechanistic studies.[85] The SD rat hepatic primary, LLC-PK1 and Hep-G2 cells are adherent, which can simplify sample preparation, and can be propagated in a 96-well plate format suitable for high-throughput screening. The 96-well format allows for detailed concentration–response curves and multiple controls to be run on the same microplate. These cell lines were subjected to a variety of in vitro assays, described below, to evaluate cytotoxicity, mechanistic toxicology, and pharmacology. These assays have been selected primarily for their superior performance, convenience, and adaptability in evaluating this new class of biomedical agent.

7.3.3 Cytotoxicity

Cell viability of adherent cell lines can be assessed by a variety of methods.[86] These methods fall broadly into four categories, assays that measure: (1) loss of membrane integrity; (2) loss of metabolic activity; (3) loss of monolayer adherence; and (4) cell cycle analysis. Data generated using these various viability assays can be used to identify cell lines susceptible to nanoparticle toxicity and potentially give clues as to the type (i.e., cytostatic/cytotoxic) and location of cellular injury. Many of the cytotoxicity assays discussed below are available as commercial kits. These kits should be used whenever feasible since they provide an extra level of quality control.

1. Membrane integrity assays are particularly important as a measure of cellular damage, since there is evidence that some cationic nanoparticles, such as amine terminated dendrimers, exhibit toxic effects by disrupting the cell membrane.[87] Examples of assays that measure membrane integrity include the trypan blue exclusion assay and lactate dehydrogenase (LDH) leakage assay, which measures the presence of LDH released into the media through cell lysis.[88,89] The LDH leakage assay was selected because of its sensitivity and suitability for the high-throughput, 96-well plate format.
2. Examples of assays which measure metabolic activity include tetrazolium dye reduction, ATP, and 3H-thymidine incorporation assays. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay was chosen for measurement of metabolic activity in the assay cascade, since it does not use radioactivity, and historically has been proven sensitive and reliable. MTT is a yellow water-soluble tetrazolium dye that is metabolized by live cells to water insoluble, purple formazan crystals. The formazan can be dissolved in DMSO and quantified by measuring the absorbance of the solution at 550 nm. Comparisons between the spectra of samples from nanoparticle treated and untreated cells can provide a relative estimate of cytotoxicity.[90]

   The MTT assay requires a solubilization step that is not required for the newer generation of tetrazolium dyes that form water-soluble formazans (e.g., XTT). However, these analogs require an intermediate electron acceptor that is often unstable, adding to assay variability. Furthermore, the net negative charge of these newer analogs

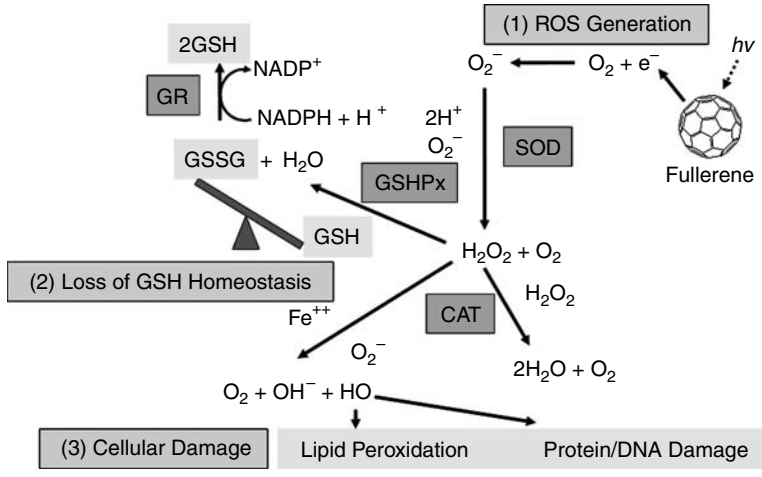
limits cellular uptake, resulting in extracellular reduction.[91] MTT, with a net positive charge, readily crosses cell membranes and is reduced intracellularly, primarily in the mitochondria. Because nanoparticles have been shown to interact with cell membranes and could potentially interfere with the reduction of the newer generation analog via trans-plasma membrane electron transport, the traditional MTT assay would appear to be a better choice to assess cellular viability in nanoparticle cytotoxicity experiments. Analytes that are antioxidants, or are substrate/inhibitors of drug efflux pumps, have been shown to interfere with the MTT assay.[92,93] Functionalized fullerenes, which have not identified as efflux pump inhibitors or substrates, but do possess potent antioxidant activity, have been observed in our laboratory to cause MTT assay interference, resulting in enhanced MTT reduction and overestimation of cell viability (unpublished data).

3. Loss of monolayer adherence to plating surfaces is often used as a marker of cytotoxicity. Monolayer adherence is commonly measured by staining for total protein, following fixation of adherent cells. This simple assay is often a very sensitive indicator of loss of cell viability.[55] The sulforhodamine B total protein staining assay was selected for determination of monolayer adherence. Advantages of this assay include the ability to store the fixed, stained microplates for extended periods prior to measurement, making the assay especially suitable for high throughput.[94]
4. Cell cycle analysis is conducted using propidium iodide staining of DNA and flow cytometry.[95] Flow cytometric can be used as a screening test for toxicity of chemicals. This method can determine the effect of nanoparticle treatment on cell cycle progression, as well as cell death. Cell cycle effects have been shown for a variety of nanoparticles. For instance, carbon nanotubes have been shown to cause G1 cell cycle arrest in human embryonic kidney cells, with a corresponding decrease in expression of G1-associated cdks and cyclins.[96]

7.3.4 Oxidative Stress

The generation of free radicals by nanomaterials is well documented.[97,98] In most cases, the studied material was of ambient or industrial origin (quartz, carbon black, metal fumes, and diesel exhaust particles). However, engineered nanomaterials, such as fullerenes and polystyrene nanoparticles, have been shown to generate oxidative stress as well.[40,99,100] Lovric et al., for example, determined ROS to play an important role in cytotoxicity of quantum dots that have lost their protective coating.[101] The unique surface chemistries, large surface area, and redox active or catalytic contaminants (e.g., metals, quinones) of nanoparticles can facilitate ROS generation.[102] For example, fullerenes can perform electron transfer (phase-I pathway) or energy transfer (phase-II pathway) reactions with molecular oxygen following photoexcitation,[44] resulting in the formation of the superoxide anion radical or singlet oxygen, respectively. The superoxide anion radical can then undergo further reactions, such as dismutation and Fenton chemistry, to generate additional ROS species (e.g., $^{\cdot}OH$), resulting in cellular injury (see Scheme 7.1).[103] Evidence of fullerene-induced oxidative stress includes lipid peroxidation in the brains of exposed fish and treated rat liver microsomes.[40,104] Additional biomarkers of oxidative stress include a decrease in the reduced glutathione/oxidized glutathione ratio (GSH/GSSG), DNA fragmentation, and protein carbonyls.[105]

Biomarkers of nanoparticle-induced oxidative stress measured in our laboratory include ROS, lipid peroxidation products, and GSH/GSSG ratio. The fluorescent dichlorodihydroflourescein (DCFH) assay is used for measurement of ROS, such as hydrogen peroxide.[106] DCFH-DA is a ROS probe that undergoes intracellular deacetylation, followed by ROS-mediated oxidation to a fluorescent species, with excitation 485 nm and emission 530 nm. DCFH-DA can be used to measure ROS generation in the cytoplasm and cellular organelles, such as the mitochondria. The



SCHEME 7.1 (1) Photoexcited fullerenes can perform electron-transfer reactions with molecular dioxygen to form the superoxide anion radical ($O_2^{\cdot -}$). Superoxide can then undergo superoxide dismutase (SOD)-catalyzed dismutation to hydrogen peroxide (H_2O_2). H_2O_2 is a substrate for catalase (CAT)-and glutathione peroxidase (GSHPx)-catalyzed detoxification reactions. (2) The oxidation of glutathione (GSH) to form oxidized glutathione (GSSG) during detoxification of H_2O_2 can result in a loss of glutathione homeostasis. GSH can be regenerated by glutathione reductase (GR). (3) Alternatively, hydrogen peroxide can undergo transition metal (Fe^{++})-catalyzed Fenton chemistry to form the highly reactive hydroxyl radical ($HO^{\cdot}$) that is capable of initiating lipid peroxidation and DNA/protein oxidation.

thiobarbituric acid reactive substances (TBARS) assay is used for measurement of lipid peroxidation products, such as lipid hydroperoxides and aldehydes. A molondialdehyde (MDA) standard curve is used for quantitation. MDA, a lipid peroxidation product, combines with thiobarbituric acid in a 1:2 ratio to form a fluorescent adduct, that is measured at 521 nm (excitation) and 552 nm (emission). TBARS are expressed as MDA equivalents.[107] The dithionitrobenzene (DTNB) assay is used for evaluation of glutathione homeostasis. In the DTNB assay, reduced GSH interacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to form the colored product 2-nitro-5-thiobenzoic acid, which is measured at 415 nm, and GSSG. GSSG is then reduced by glutathione reductase to form reduced GSH, which is again measured by the preceding method. Pretreatment with thiol-masking reagent, 1-methyl-4-vinyl-pyridinium trifluoromethane sulfonate, prevents GSH measurement, resulting in measurement of GSSG alone.[108]

7.3.5 Apoptosis and Mitochondrial Dysfunction

Nanoparticle-induced cell death can occur by either necrosis or apoptosis, processes that can be distinguished both morphologically and biochemically. Morphologically, apoptosis is characterized by perinuclear partitioning of condensed chromatin and budding of the cell membrane to form apoptotic bodies, whereas necrosis is characterized by cellular swelling (oncosis) and blebbing of the cell membrane.[109] In vitro studies have demonstrated the ability of nanoparticles, such as dendrimers and carbon nanotubes, to induce apoptosis.[110–112] In vitro exposure of macrophage-like mouse RAW 264.7 cells to cationic dendrimers led to apoptosis confirmed by morphological observation and the evidence of DNA cleavage.[112] Pretreatment of cells with a general caspase inhibitor (zVAD-fmk) reduced the apoptotic effect of the cationic dendrimer.[112] Apoptosis has also been observed in cultured human embryonic kidney cells (HEK293) and T lymphocytes treated

with single walled carbon nanotubes, and in MCF-7 breast cancer cells treated with quantum dots.[101,110,113]

Apoptosis in mammalian cells can be initiated by four potential pathways: (1) mitochondrial pathway, (2) Death receptor-mediated pathway, (3) ER-mediated pathway, and (4) Granzyme B-mediated pathway.[114] Our laboratory has focused on caspase-3 activation in liver and kidney cells as a biomarker of apoptosis, since this a downstream event in all the classical apoptotic signaling pathways and can be measured using a fluorometric protease assay. This assay quantifies caspase-3 activation in vitro by measuring the cleavage of DEVD-7-amino-4-trifluoromethyl coumarin (AFC) to free AFC that emits a yellow-green fluorescence ($\lambda_{max} = 505$ nm).[115] This initial apoptosis screen can then be followed by additional analysis, as cellular morphology studies using nuclear staining techniques to detect perinuclear chromatin, or agarose gel electrophoresis to detect DNA laddering.[116]

Evidence supports a role for ROS in generation of the mitochondrial permeability transition via oxidation of thiol components of the permeability transition pore complex.[117] As discussed in the preceding sections, nanoparticles have been shown to induce oxidative stress, and thus this ROS-mediated pathway for induction of the mitochondrial permeability transition is a plausible apoptotic mechanism for nanomaterials. For instance, ambient ultrafine particulates have been shown to translocate to the mitochondria of RAW 264.7 murine macrophage cells, cause structural damage, and altered mitochondrial permeability.[98] A subsequent study demonstrated that mitochondrial dysfunction and apoptosis in the RAW 264.7 cells could be induced by polar compounds fractionated from ultrafine particles, suggesting that the mitochondrial dysfunction caused by ultrafines was the result of redox cycling of quinone contaminants on the surface of the particle.[118] This link between oxidative stress, mitochondrial dysfunction, and apoptosis has also been observed for man-made nanoparticles. For example, metal and quantum dot engineered nanoparticles have both been shown to induce oxidative stress, mitochondrial dysfunction, and apoptosis in various in vitro models.[101,119] Water-soluble, derivatized fullerenes, which have been shown to accumulate in the mitochondria of HS 68 human fibroblast cells, have also been shown to induce apoptosis in U251 human glioma cells.[120,121] While this derivatized fullerene-induced apoptosis in the glioma cell line did not involve oxidative stress, mitochondrial dysfunction was not measured and cannot be ruled out. Mitochondrial dysfunction and apoptosis have also been observed in a human gastric carcinoma cell line exposed to chitosan nanoparticles.[122] Taken together, these observations support a role for mitochondrial dysfunction and oxidative stress in nanoparticle-induced apoptosis. Apart from apoptosis, mitochondrial dysfunction has long been associated with necrotic cell death, and represents a potential necrotic mechanism of nanoparticle-induced injury as well.[123]

Mitochondrial dysfunction can result from several mechanisms in addition to opening of the permeability transition pore complex, including uncoupling of oxidative phosphorylation, damage to mitochondrial DNA, disruption of the electron transport chain, and inhibition of fatty acid β-oxidation.[124] Methods used to detect mitochondrial dysfunction include measurement of ATPase activity (via luciferin–luciferase reaction), oxygen consumption (via polarographic technique), morphology (via electron microscopy), and membrane potential (via fluorescent probe analysis).[125] Our laboratory measured loss of mitochondrial membrane potential in rat hepatic primaries, and Hep-G2 and LLC-PK1 cell lines, using the 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) assay, which is a convenient assay that does not require mitochondrial isolation or use specialized equipment.[126] This fluorescent dye partitions into the mitochondrial matrix as a result of the membrane potential. Concentration of JC-1 in the matrix results in aggregation that fluoresces at 590 nm (red). Upon loss of membrane potential, the dye dissipates from the matrix and can be measured, in its monomer state at emission 527 nm (green). The proportion of green to red fluorescence reflects the degree of mitochondrial membrane depolarization.

7.3.6 Proteomics and Toxicogenomics

Proteomics and toxicogenomics are useful tools for identifying the mechanisms underlying toxicity.[127] Using gel electrophoresis in combination with MS identification, or gene microarray technology, the expression of specific pathway-responsive genes, such as Phase-II enzymes for oxidative stress, or cytokines for inflammation, can be identified. The delineation of these toxic pathways could help further refine the in vitro and in vivo study of nanomaterials, potentially leading to the development of novel biomarkers that could then be used in clinical and occupational toxicology studies. Proteomic and genomic research on biomedically relevant nanomaterials is presently underway, using a series of human hepatocyte, kidney, and immunological primary cells.

7.4 IN VIVO PHARMACOKINETIC AND TOXICOLOGICAL ASSESSMENTS

As is the case with any NCE, a thorough understanding of the properties that govern biocompatability is necessary to allow transition of nanomaterials to human clinical trials. Although in vitro toxicology studies can be informative, the obvious caveat is that phenomenon observed in vitro may not materialize in vivo due to differences in biological response or nanoparticle concentrations. Therefore, nanoparticle safety and therapeutic efficacy can only be definitively assessed by rigorous in vivo testing. This phase is guided in part by insights obtained from the physicochemical and in vitro characterization programs.

The primary goal of in vivo studies is to evaluate nanomaterials' pharmacokinetics, safety, and efficacy (see Table 7.1) in the most appropriate animal models. Preclinical toxicological and pharmacokinetic studies are conducted in accordance with the FDA regulatory guidance for IND and IDE submission. It is not within the scope of this chapter to review this regulatory guidance; instead, the reader is directed to the regulatory chapter of this text. While it is generally agreed that the current in vivo pharmacological and toxicological endpoints used for devices and small molecule drugs should be appropriate in assessing the safety and efficacy of biomedical nanomaterials, the qualities of nanomaterials that lend themselves to biomedical application, such as

TABLE 7.1
In Vivo Pharmacological and Toxicological Assessment of Nanomatersials

Category	Assessment
Initial disposition study	Tissue distribution
	Clearance mechanisms
	Half-life
	Systemic exposure (plasma AUC)
Immunotoxicity	28-day screen
	Immunogenicity (repeat-dose toxicity study)
	Hypersensitivity
	Immunostimulation
	Immunosuppression
Dose-range finding toxicity	NOAEL
	STD10
Good laboratory practices studies	PK/ADME
	Expanded single dose acute toxicity
	Repeated dose toxicity
Efficacy	Targeting
	Therapeutic
	Imaging

macromolecular structure and polydispersity, could also be problematic with respect to preclinical characterization, as will be discussed below. Early efforts are focused on identifying and standardizing the analytical and toxicological methodologies that are unique to nanoparticle preclinical characterization.

Several factors can influence the clinical viability of a new cancer diagnostic or therapeutic agent, aside from economic feasibility. These include: (1) demonstrated advantage over the current market standard; (2) appropriate administration route/schedule/elimination half-life; and (3) favorable safety profile.

1. The in vivo characterization phase includes an assessment of nanoparticle imaging and/or therapeutic efficacy in animal models that most closely approximate the human disease state. For instance, targeting efficacy is addressed by comparing a nanoparticle distribution profile with that of a nontargeted nanoparticle from the same class; however, this approach may be prone to ambiguities due to passive targeting via the EPR effects. For those particles with imaging components, the signal enhancement and tissue distribution profile is monitored using the appropriate magnetic resonance, ultrasound, optical, or positron emission tomography imaging instrumentation. In all cases, the approach will be compared against the present market standard to provide comparable data regarding efficacy, pharmacokinetics, and safety.
2. Animal studies of nanoparticles have rarely utilized the oral administration route, but those that have used that route have demonstrated poor intestinal absorption. For example, 98% of orally administered, PEG-functionalized fullerenes are eliminated in the feces within 48 h. Oral bioavailability of polystyrene nanoparticles were similarly poor, with less than 7% of 3000-, 1000-, 100-, 50-, and 3-nm sized particles absorbed.[128] Due to this extremely low nanoparticle oral bioavailability, the majority of biomedical nanoparticle formulations encountered undoubtedly will be intended for parenteral administration. Since diagnostic and chemotherapeutic regimens are typically of short duration, the inconvenience of intravenous administration does not appear to be a significant hurdle for eventual clinical transition.

   To be a successful diagnostic or therapeutic agent, nanoparticles should be eliminated from the body in a reasonable timeframe. However, studies have shown that optimum passive targeting of tumors, by the EPR effect, also requires that nanoparticle agents remain in the systemic circulation for prolonged periods.[129] Therefore, there must be a balance between systemic residence and clearance. At present, avoidance of the RES system and eventual urinary clearance appears to be a formidable obstacle for many of the current approaches, such as iron oxide MRI contrast agents and lipidic nanoparticle drug delivery agents, which have been shown to undergo capture by organs of the RES and remain for extended periods.[130,131] The primary mechanism of nanoparticle clearance, as discussed previously, appears to be glomerular filtration, which is governed by charge, molecular weight, and degree of protein binding.[132,133] A good case study of the importance of molecular weight in mediating glomerular filtration, by Gillies and colleagues, demonstrated that dendrimer–polyethylene oxide complexes greater than 40 kDa were cleared less readily than lower molecular weight species. Because timely clearance is an important drug attribute, accurate and thorough disposition studies are required.

   The single greatest obstacle for nanoparticle disposition studies is analytical methodology to quantitate nanoparticle concentrations in biological matrices, such as plasma and tissues. Due to their macromolecular and polydispersed nature, nanomaterials do not lend themselves to quantitation by traditional methods, such as HPLC and LC/MS, and may require alternative methods such as radiolabeling and scintillation

counting. Since many biomedical nanoparticles will be multifunctional, and have imaging components, the imaging functionality of the particle could potentially be used for in vivo quantitation. In cases where no imaging component is present, the nanoparticles could be tagged with an appropriate probe to allow for imaging quantitation. The labeling method utilized may alter the surface properties of a nanoparticle, and thus affect the tissue distribution profile; comparison of alternative labeling methods would help identify consensus behavior. In any event, the use of imaging would first require validation using traditional methods such as LC/MS or scintillation to ensure image intensity could correlate to nanoparticle concentration in a linear fashion. Since many nanomaterials are electrondense, electron microscopy might also be used for tracking tissue distribution.

3. The objectives of the preclinical toxicological studies are to identify target organs of toxicity and to aid in the selection of starting doses for phase-I human clinical trials. Toward this end, toxicity studies seek to determine dose ranges causing (1) no adverse effects (NOAEL) and (2) life-threatening toxicity (i.e., severe toxic dose 10% [STD_{10}]). Studies are performed in two mammalian species, rodent and nonrodent, with rats the preferred rodent species, since they exhibit the greatest concordance with human toxicities.[134] Nanoparticle formulations are administered according to the intended clinical treatment cycle, with regard to schedule, duration, route, and formulation. Necropsy, performed on animals showing signs of morbity during the study and at study termination, includes comprehensive hematology, histopathology, and clinical chemistry (see Figure 7.3). Several preclinical studies suggest a key role for reticuloendothelial organs, such as liver, kidney, and bone marrow, in the uptake of nanoparticles from the systemic circulation.[67,69] Therefore, these organs should receive special attention with regard to functional and histopathological evaluation. A review of the limited in vivo safety data available for nanoparticles supports the scrutinizing of these tissues, as there are several examples of RES organ injury, including hepatotoxicity and nephrotoxicity. For example, intravenous administration of cationic PAMAM dendrimers at low doses has

Histopathology

Brain	Pancreas	Salivary gland
Lymph node	Esophagus	Parathyroid
Thyroid	Trachea	Adrenal
Pituitary	Heart	Kidney
Thymus	Gall bladder	Liver
Spleen	Lung	Duodenum
Ileum	Rectum	Stomach
Cecum	Colon	Jejunum
Lymph node	Epididymis	Ovary
Prostate	Seminal vesicle	Testis
Urinary bladder	Uterus	Eye
Hardian gland	Nasal sections	Femur
Femur	Vertebra	Spinal cord
Mammary gland	Skin/subcuits	Tongue

Hematology

Erthrocyte count (RBC)
Hemoglobin (HGB)
Hematocrit (HCT)
Mean corpuscular volume (MCV)
Mean corpuscular hemoglobin (MCH)
Mean corpuscular hemoglobin conc. (MCHC)
Platelet count (Plate)
Reticulocyte count (RETIC)
Total leukocyte count (WBC)
Differential leukocyte count
Nucleated red blood cell count

Clinical Chemistry

BUN	AST	ALT
GGT	GLUC	Creatinine
total protein	Albumin	Globulin
A/G	Sodium	Potassium
Chloride		

FIGURE 7.3 Panel of tissues and chemistries to be assessed for comprehensive toxicology.

been shown to cause liver injury, as determined by histopathology and elevations in serum alanin aminotransferase when administered intravenously to mice; larger doses of the same cationic dendrimer were lethal to 100% of the mice.[135] Nephrotoxicity and hepatotoxicity, as determined by histopathology and serum enzyme markers, have both been observed in mice treated orally with nano-zinc.[72,84]

One of the potential advantages of nanoparticle drug formulations is an improved safety profile as a result of targeted therapy or elimination of toxic solubilization agents. For example, Baker and colleagues have shown that methotrexate-conjugated PAMAM dendrimers containing a folate receptor targeting ligand are more efficacious, and less toxic, than unformulated methotrexate against a murine human epithelial cancer model.[34] Abraxane is an example of a nanoparticle formulation of paclitaxel, presently on the market, that takes advantage of the solubilizing effect of albumin, eliminating the need for the toxic vehicle Cremophor EL.[136] Phase-III studies have shown enhanced therapeutic response of abraxane compared to Cremophor-formulated paclitaxel, while side effects, such as myelosuppression and peripheral neuropathy, were significantly reduced. As discussed above, nanoparticle drug formulations are compared against the unformulated drug to determine if the improvement in safety profile is realized.

7.5 IMMUNOTOXICITY

A growing body of evidence suggests that immunotoxicity provides a considerable contribution to onset and development of various disorders, including cancer and autoimmune diseases.[137–139] Nevertheless, it was not until recently that this relatively new field of toxicology emerged as an important interface between the fields of novel drug design and pharmacology. Recognition of immunosuppressive properties of new pharmaceuticals during early drug development phase is very important to eliminate potentially dangerous substances from the drug pipelines. For example, treatment of patients diagnosed with Crohn's disease and rheumatoid arthritis with infliximab and etanercept (both drugs represent neutralizing anti-TNF antibodies) resulted in increased incidence of tuberculosis and histoplasmosis.[140–143] Although these data did not result in withdrawal of any of the products from the pharmaceutical market, they helped initiate the strategy of preparing patients for anti-TNF therapy by screening for, and treatment of, latent tuberculosis prior to administration of anti-TNF medications. Immunosuppression caused by pharmaceuticals can also lead to the development of lymphomas and acute leukemia.[144–146] Undesirable immunostimulation caused by pharmacological intervention include immunogenicity, hypersensitivity, and increased risk of autoimmune response. The standard toxicology endpoints employed for safety assessment of new pharmaceuticals primarily rely on clinical chemistry and histopathological evaluation of immune organs and were developed several decades ago. Currently, there is an increasing demand for the development of new methods for immunotoxicity assessment because of drug candidates' more complex structure as well as the application of new technologies in their manufacturing. The introduction of new molecular and immune cell biology methods into the immunotoxicology assessment framework is not a trivial and straightforward process. It requires not only scrupulous validation and standardization of the new techniques but also demonstration of the physiological relevance for the proposed battery of assays. These processes are expensive, time-consuming, and necessitate cooperation across the various pharmaceutical industry players. Unlike traditional drugs, multifunctional nanomaterials combine both chemistry-based and biotechnology-derived components, and therefore their characterization using standard methodologies requires adjustments and/or modification of classical experimental protocols. Below we will attempt to summarize data on critical aspects of immunotoxicological evaluation of nanomaterials and examine challenges in the application of standard methodologies for the assessments of nanoparticle safety to the immune system.

7.5.1 Applicability of Standard Immunological Methods for Nanoparticle Evaluation and Challenges Specific to Nanoparticle Characterization

Immunotoxicological evaluation of new drug candidates includes studies on both immunosuppression and immunostimulation, and is applicable to nanomaterials intended for use as drug candidates and/or drug delivery platforms. Short-term in vitro assays are being developed to allow for quick evaluation of nanoparticles' biocompatibility. The in vitro immunotoxicity assay cascade includes the following methods: analysis of plasma protein binding by two-dimensianol polyacrylamide gel electrophoresis (PAGE), hemolysis, platelet aggregation, coagulation, complement activation, colony forming unit-granulocyte macrophage (CFU-GM), leukocyte proliferation, phagocytosis, cytokine secretion by macrophages, chemotaxis, oxidative burst, and evaluation of cytotoxic activity of NK cells.[147] In addition to these methods, our in vitro tests include sterility assessment based on pyrogen contamination test (L-amebocyte lysate (LAL) assay) and evaluation of microbiological contamination. The assay cascade is based on several regulatory documents recommended buy the FDA for immunotoxicological evaluation of new investigational drugs, medical devices, and biotechnology, derived pharmaceuticals,[148–152] as well as on ASTM and ISO standards developed for characterization of blood contact properties of medical devices.[153–155] The aim of the in vitro immunoassay cascade is to provide quick evaluation of nanomaterials of interest prior to initiation of more thorough in vivo studies. Challenges specific for immunotoxicity assessment of nanoparticulate materials are summarized below.

7.5.1.1 Blood Contact Properties

One important aspect of nanoparticle used for medical applications is the assurance that they will not cause toxicity to blood elements when injected into a patient.

Hemolysis (i.e., damage to red blood cells) can lead to life-threatening conditions such as anemia, hypertension, arrhythmia, and renal failure. In our laboratory we have developed a protocol to evaluate hemolytic properties of nanoparticles based on the existing ASTM International standard used to characterize other materials.[147,156] We have identified several problems when applying existing protocol for nanoparticles characterization. For example, colloidal gold nanoparticles with size 5–50 nm have absorbance at 535 nm, which overlaps with the assay wavelength of 540 nm. Removal of these particles by centrifugation was required prior to sample evaluation for the presence of plasma-free hemoglobin to avoid false-positive results. Though it worked well for gold particles with size 10–50 nm, centrifugation may be problematic for other nanoparticles. For example, small colloidal gold particles with a size of 5 nm require higher centrifugation force to be removed from the supernatant. Hemoglobin has a size of 5 nm; therefore, one cannot exclude the possibility that ultracentrifugation of supernatant may pellet hemoglobin along with the gold particles and thus result in a false-negative result. Ultracentrifugation is not feasible for fullerenes or dendrimer particles. Analysis of polystyrene particles of 20, 50, and 80 nm revealed another complication. We found that particle preparation damages red blood cells; the damage is caused by the surfactant used during particle manufacturing, and is detected for 20- and 50-nm particles. For 80-nm particles, the hemolysis assay showed false-negative results due to the adsorption of hemoglobin by the particles. When we applied dialysis to remove surfactant, 50-nm particles revealed same phenomenon as 80-nm particles, i.e., they caused hemolysis, but adsorbed hemoglobin resulting in a false-negative result. Another potential problem with the application of standard hemolysis protocol for nanoparticles characterization is that metal-containing particles may oxidize hemoglobin and result in a change in assay OD responses. Therefore, assay procedures may require slight modifications depending on the particle type. In general, inclusion of particle test samples without blood allowed for quick assessment of the potential particle interference with the assay. In some instances, deduction of the result generated for blood-free particle control from that

obtained for blood-plus particle sample was possible and allowed estimation of particle potential to cause damage to erythrocytes.

Blood coagulation. Blood coagulation may be affected by nanomaterials. For example, modification of surface chemistry has been shown to improve immunological compatibility at the particle–blood interface: application of polyvinyl chloride resin particles resulted in $19 \pm 4\%$ decrease in platelet count, indicating platelet adhesion/aggregation and increased blood coagulation time; the same particle preparation coated with PEG affected neither the platelet count nor elements of coagulation cascade.[157] Similarly, folate-coated and PEG-coated Gd nanoparticles did not aggregate platelets or activate neutrophils.[158] The evaluation of nanoparticle effects on blood coagulation includes studies on platelet function and coagulation factors. The in vitro cascade includes platelet aggregation assay and four coagulation assays measuring prothrombin time, activated partial thromboplastin time, thrombin time, and reptilase time.

Interaction with plasma proteins. High-resolution, 2D PAGE may be the method of choice to investigate plasma protein adsorption by nanoparticles. 2D PAGE has been used in several labs to isolate and identify plasma proteins adsorbed on the surface of stealth polycyanoacrylate particles[8], liposomes[159,160], solid lipid[161], and iron oxide nanoparticles.[162] Proteins commonly identified on several types of nanomaterials include antithrombin, C3 component of complement, alpha-2-macroglobulin, haptoglobin, plasminogen, immunoglobulins, albumin, fibrinogen, apolipoprotein, and transthyretin; albumin, immunoglobulins, and fibrinogen are the most abundant. Studies using this approach revealed that surface chemistry is important for protein adsorption. For example, Peracchia et al. demonstrated that coating with PEG results in approximately a fourfold reduction in protein binding by polycyanoacrylate particles.[163] Gessner et al. prepared polystyrene latex model nanoparticles with different surface charges. This study demonstrated that increasing surface charge density results in a quantitative increase in plasma protein adsorption, but did not show significant differences in the qualitative composition of the absorbed protein mixture.[164]

One of the most important step in this procedure is the separation of particles from plasma after incubation is complete. Ultracentrifugation was shown to be successful for isolation of iron oxide, solid lipid particles, and some polymer-based particles.[8,160,162] Gel filtration was applicable to liposomes,[159] solid lipid, and iron oxide nanoparticles. Thode and colleagues compared four methods for isolation of iron oxide particles, i.e., ultracentrifugation, static filtration, magnetic separation, and gel filtration. Depending on the method used for particle separation from bulk plasma, different quantities of the same proteins and different species of proteins were identified on the particles of the same size and surface chemistry.[162] For example, albumin was the predominant protein if static filtration and gel filtration were employed, while small quantities of this protein were found after ultracentrifugation; it was almost undetectable when magnetic separation was used. There was no difference in isolation of fibrinogen among the four methods. Comparable quantities of IgG gamma-chain were isolated using ultracentrifugation, static filtration, and magnetic separation, while gel filtration appeared to be inefficient in isolation of this protein.[162] Attention has to be paid to the sample preparation to avoid artificial protein adsorption due to desorption during the separation, for example. Other critical steps are the number of washes to remove an excess of bulk plasma, the type of wash buffer, and a buffer to dislodge protein from the particle surface. In our lab we also found that using polypropylene low-retention tubes and pipette tips is crucial for isolation of particle-specific proteins (unpublished data).

Complement activation and phagocytosis. Following intravenous administration, nanoscale drug carriers may suffer a drawback in that they may be taken up by cells of the mononuclear phagocytic system. Consequently, such uptake facilitates clearance of nanoparticulate carriers and associated drugs, thus leading to a decrease in drug efficacy.[165] The initial adsorption of plasma proteins such as components of complement and immunoglobulins promotes nanoparticles clearance.[166,167] Therefore, the investigation of a nanoparticle's ability to interact with and activate a complement and uptake by mononuclear cells seems to be one of the key assays in the preclinical characterization cascade. Classical immunoassays used to evaluate complement activation, such as

the total hemolytic complement assay (CH50) and the alternative pathway (rabbit CH50 or APCH50), are based on the hemolysis of rabbit erythrocytes. These hemolytic assays can be used to measure functional activity of specific components of either pathway. The main challenge in applying these assays for nanomaterial characterization is the ability of nanoparticles per se to lyse RBCs, thus generating false-positive results. To overcome this limitation, the approach for evaluation of complement activation includes two techniques. One is a qualitative yes or no rapid screen for the presence of C3 cleavage products using western blot. The second assay is a quantitative evaluation of samples found positive at an initial screen for the presence of C4a, C3a, and C5a components of complement using a flow cytometry-based multiplex array.

The difficulties with application of standard phagocytosis assay are: (1) light microscopy used in traditional phagocytosis assay[168] is not applicable to nanoparticles due to their smaller size; (2) when light microscopy is substituted with TEM, visualization of particle may be complicated since their size is similar to that of cell organelles resulting in ambiguous interpretation of TEM data, thus TEM is limited to electron dense metal containing particles (see Figure 7.4); (3) labeling of nanoparticles with fluorescent tags[9,169] is a superior approach for visualizing internalized particles, but should be avoided in preclinical tests as chemical attachment of fluorophore may create a new molecular entity with properties widely divergent from those of the original particle; (4) application of luminol to detect phagocytosed particles[8] provides an exceptional technique which can overcome all limitations listed above; however, it is not free of applicability reservations as well (e.g., nanoparticles may interfere with activation of luminol once it is internalized, etc.).

CFU-GM assays. CFU-GM assays allow for the evaluation of potential nanoparticles interference with growth and differentiation of bone marrow stem cells into granulocyte and macrophages. This assay may provide valuable information for development of anti-cancer nanotechnology platforms. Myelosuppression is a very common dose-limiting toxicity associated with the use of oncology cytotoxic drugs. Incorporation of such drugs into nanoparticle carriers targeted to specific cancer cells may help to reduce toxicity to normal tissues, including bone marrow, and should be considered during initial characterization of nanocarriers.

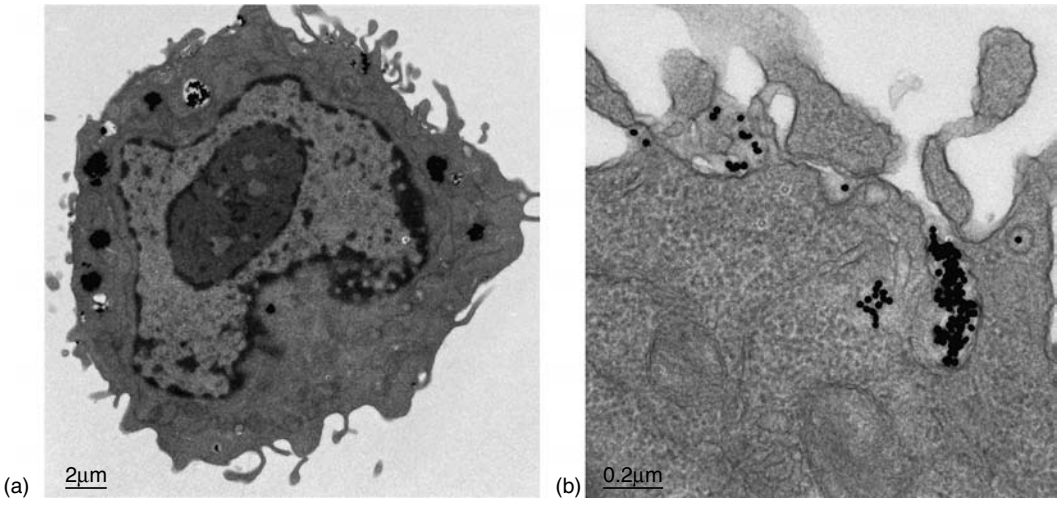


FIGURE 7.4 Study of internalization of 30-nm colloidal gold nanoparticles by murine macrophage cell line RAW 264.7 by TEM. (a) Analysis of single cell. (b) Zoom-in analysis of the selected area in the same cell.

7.5.2 Immunogenicity

This issue of immune stimulation by pharmaceuticals came into the forefront when biotechnology-derived products, especially recombinant proteins, moved toward clinical trials. Today it is evident that the immune system can effectively recognize biological therapeutics as foreign substances and build up a multi-level immune response against them. A number of factors result in the immune system responding to the administration of a pharmacological product, such as structure, formulation, folding architecture, but also degradation byproducts.[170] In addition, the route of administration and the dosage were shown to influence the staging and the amplitude of the immune system's response. In general, immune responses to biological products could be classified as benign in the sense that they affect only the pharmacological efficacy of the administered compound. The greatest concern is the robust immune response to certain biotechnology-derived products that are fraught with serious clinical consequences that may even result in a fatal outcome due to specific recognition and elimination of the patient's endogenous growth factors critical for survival.[171–173] For example, in the case of thrombopoietin, the immune response may result in the production of neutralizing antibodies, causing inhibition of the endogenous thrombopoietin with subsequent development of thrombocytopenia.[173] The patient's immune response to recombinant erythropoietin product Eprex® has been reported to induce pure red cell aplasia.[171,172,174] In the latter example, cross-reactivity tests indicated that antibodies generated against Eprex® could also neutralize other forms of erythropoietin products such as Epogen®, NeoRecormon®, and Aranesp® and suggested that antibodies are directed against some specific conformation of the erythropoietin active site.[170] Although incidence of the acute pure red cell aplasia remains relatively rare, the long-term implications are of great concern, as over half of patients who developed the auto-antibody remained transfusion dependent. The potential of using multifunctional nanoparticles for medical applications raises a key question of whether nanoparticle materials by themselves can induce an anti-nanoparticle immune response, stimulate allergic reactions, or trigger synthesis of nanopreparation-specific IgE. One can expect that the generation of antibodies to nanoparticles will ultimately affect only efficacy of the particle-based product. Of greatest concern will be the immune response to particles functionalized with growth factors, receptors or other biological molecules, which would result in the formation of antibodies, neutralizing the effect of these biological molecules and leading to potential exclusion of both particle-linked and endogenous proteins, akin to similar effects observed with biotechnology-derived pharmaceuticals. There are a limited number of studies on immunogenicity of nanomaterials. A few of them have shown that nanoparticles may both induce nanoparticles specific immune response and act as adjuvants.[175–178] Although preclinical animal studies may not be predictive to human immune response, available data described above do suggest that the immune system can recognize and build an immune response against some nanoparticles. Therefore, evaluation of nanoparticle antigenicity is seen as an important step during preclinical development. Other immunogenicity characterization should include evaluation of a nanoparticles' ability to act as an adjuvant and to induce allergic reactions.

7.6 CONCLUSIONS

The urgency to eliminate the pain and suffering associated with cancer is fueling research into novel therapies at a blistering pace. Through exquisitely targeted and multifunctional approaches, nanotechnology in particular holds great promise for enhancing cancer therapy. This promise, however, will never be realized if the safety of the nanomaterial is not demonstrated to allay public concern and if the regulatory structure is not in place to allow proper evaluation of the science. Without such a framework, the return on investment will be uncertain and progress will undoubtedly be stalled. The effort to develop a standardized set of protocols to characterize nanomaterials and their biological effects will provide a foundation for future regulation and will lead to a body of knowledge that will guide the design of safer nanotechnology products.

The biologic activity and toxicity of nanoscale particles are dependent on many parameters not typically examined for conventional small molecule therapeutics: size, shape, surface chemistry, stability of outer coating, agglomeration state, etc., and many conventional properties, such as stability or biodistribution, must be analyzed using a very different set of protocols and/or instrumentation. Emerging data from studies on nanoparticles engineered for medical use will build toward a consensus of instrumentation and experimental methodology needed to reproducibly determine the pharmacology and safety of these novel products.

The greatest challenge may not be in the development of new screening technologies, but in the ability to promulgate an accepted set of characterization protocols throughout the Nano Bio industry. The Nanotechnology Characterization Laboratory at the National Cancer Institute is working together with the FDA, NIST, and other regulatory and standards-setting organizations to establish the standard assays and technologies needed for timely delivery of safe and effective nanotechnology products.

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