

# Chapter 2

## Challenges for Nanoparticle Characterization

Scott E. McNeil

### Abstract

The Food and Drug Administration (FDA) and pharmaceutical industry have used standards to assess material biocompatibility, immunotoxicity, purity, and sterility (as well as many other properties) for several decades. Nanoparticle developers and manufacturers leverage well-established methods as much as possible. However, the unique properties of nanomaterials often interfere with standardized protocols, giving false-positive or false-negative results. This chapter provides details of some of the problems which can arise during the characterization of nanoparticle samples. Additionally, we discuss ways to identify, avoid, and resolve such interference, with emphasis on the use of inhibition and enhancement controls.

**Key words:** Nanoparticles, nanomedicine, active and passive targeting, efficacy, toxicity

Nanotechnology offers the potential to significantly transform diagnostics and therapeutics, as described in the previous chapter. The ability to manipulate the biological and physicochemical properties at the macromolecular size-scale allows for efficient drug targeting and delivery, which result in greater potency and decreased adverse side effects. Nanoparticles intended for clinical applications consist of a wide variety of materials, for which preclinical characterization is particularly challenging. Many of these particles scatter light (e.g., gold colloids) or have optical properties which may invalidate colorimetric assays that rely on absorbance measurements (e.g., quantum dots). Other nanoparticles, such as dendrimers, can have catalytic properties that interfere with enzymatic tests.

Most nanoparticle formulations include surfactants to promote dispersion (i.e., prevent agglomeration) of the primary particles. These compounds too can interfere with conventional characterization methods. Impurities and contaminants which adsorb to nanoparticle surfaces can also contribute to ambiguous analytical results. These difficulties tend to hamper the development of standards for characterization and the subsequent clinical application of nanoparticles.

An investigational new drug (IND) or investigative device exemption (IDE) application is the first step in the FDA approval

process which is required by law before a developer can test a candidate drug's therapeutic or diagnostic potential in humans (1). Preclinical testing data in the IND must demonstrate that the new drug will not expose humans to unreasonable risks during initial use, and, in the case of therapeutics, that the drug exhibits sufficient pharmacological activity to justify first-in-man clinical trials. For small-molecule drugs, the FDA has criteria for the types of preclinical data which should be presented in an IND. For nanomaterials, an IND can be less straightforward, since there is no standardized set of characterization methods for these materials. Until such standards become available, nanotech developers have to design and validate their own novel characterization methods to assess safety, toxicity, and quality control. The FDA then faces the difficulty of interpreting data generated by a variety of unfamiliar techniques without a substantial history of acceptance in scientific literature. All of this complicates the preclinical development process and can increase the time preceding first-in-man trials for nanotech-based drugs.

One of the chief complications for preclinical characterization is the multicomponent nature of many nanoparticle-based therapeutics. The nanoparticle can serve as a scaffold for attachment of chemical moieties that each perform a particular medical function (e.g., targeting ligands, hydrophilic coatings that improve solubility, imaging agents, drugs, etc.). The resulting nanoparticle therapeutic is a multipart, multifunctional entity with greater complexity than a conventional small-molecule drug. Assessing the safety and efficacy of such a complex entity can be a daunting task. Ultimately, the realization of the use of these multicomponent nanoparticles in clinical trials is highly dependent on rigorous preclinical characterization.

Thorough characterization is also key for evaluating the safety of nanoparticles for incidental exposure and addressing concerns about environmental health and safety (EHS). Whether or not nanomaterials are more toxic than their macroscale counterparts has been a matter of extensive debate in the EHS community. The scientific literature contains a wide range of research findings, which are often conflicting due to the variety of methods used and to subtle variations in test materials. Arriving at a definitive answer to this question will depend on thorough characterization using standardized methods and materials.

A rational characterization strategy for biomedical nanoparticles contains three elements: physicochemical characterization, in vitro assays, and in vivo studies. Each of these is essential to a comprehensive understanding of nanoparticle safety and efficacy. For example, without physicochemical characterization there can be no meaningful interpretation of in vitro or in vivo biological data or interlaboratory comparison. The simplicity and amplified reactions of in vitro assays may help elucidate the biological

mechanism of action of a therapeutic or toxicant. Testing in *in vitro* physiological models can also give an initial estimate of formulation efficacy and toxicity. Realistically though, it is not possible for the laboratory bench to exactly match the complex biological interplay found *in vivo*. It is therefore necessary to characterize the absorption, distribution, metabolism, and excretion and toxicity (ADME) of a drug formulation in animal models.

In terms of physicochemical properties, traditional small-molecule drugs are characterized by their molecular weight, chemical composition, purity, solubility, and stability. These data form the basis of the chemistry, manufacturing, and controls (CMC) section of the IND application with the FDA. For small molecules, the instrumentation to ascertain these properties have been well established and the techniques are standardized. Techniques like nuclear magnetic resonance (NMR), mass spectrometry, ultraviolet-visible (UV-Vis) spectroscopy, infrared spectroscopy (IR), and gas chromatography (GC) can be run in a high-throughput fashion to analyze such molecules. For nanomaterials, alternate instrumentation is required to obtain information on the same properties (composition, purity, stability, etc.). These properties influence biological activity, and may depend on parameters such as particle size, size distribution, surface area, surface charge, surface functionality, shape, and aggregation state. Additionally, since many nanoparticle concepts are multifunctional (with targeting, imaging, and therapeutic components), the stability and distribution of these components can have dramatic effects on nanoparticle biological activity as well.

It is now widely acknowledged that physicochemical properties such as size and surface chemistry can dramatically affect nanoparticle behavior in biological systems (2–7) and influence biodistribution, safety and efficacy. For instance, a decrease in particle size leads to an exponential increase in surface area per unit mass, and a concomitant increase in the availability of reactive surface groups. Nanoparticles with cationic surfaces have a notably increased tendency to permeate (and perforate) cellular membranes compared to neutral or anionic nanoparticles (8). Physicochemical characterization of properties such as size, surface area, surface chemistry, and aggregation state can provide the basis for better understanding of structure–activity relationships. In this volume, methods are presented for determining nanoparticle size in solution by dynamic light scattering (DLS), molecular weight via mass spectrometry, surface charge through zeta potential measurement and topology by atomic force microscopy (AFM). Methods are also presented for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) examination of nanoparticle samples, and elemental identification using energy dispersive X-ray spectroscopy (EDX).

Another important and challenging area of nanoparticle characterization is measurement under physiological conditions that resemble or mimic the physical state *in vivo*. Many properties of nanoparticles are environment and condition dependent; for example, the particle's hydrodynamic size at physiological pH and ionic strength may differ from the size in water or the dry state. Surface charge may also depend on the pH and ionic strength of the suspending solution. Plasma proteins are known to bind nanoparticles in the blood, and the protein-bound size is expected to be a more relevant determinant of disposition and clearance than the free-particle size. The release profile of an encapsulated therapeutic may even be environment dependent (9). That is, there may be faster or slower release of the therapeutic as the temperature, pH, and/or ionic strength of the solution surrounding the nanoparticle formulation is varied.

Because the results of *in vitro* biological assays often don't correlate with *in vivo* endpoints, *in vitro* characterization is performed to elucidate mechanisms, not necessarily to screen for biocompatibility. *In vitro* studies may also be used to identify areas requiring attention for *in vivo* animal studies. Unfortunately, nanoparticle-based therapeutics frequently interfere with conventional *in vitro* pharmacologic assays. For instance, many nanoparticles aggregate or adsorb proteins. Other nanoparticles scatter light or have optical properties, which may invalidate colorimetric assays that rely on absorbance measurements. Some nanoparticles have catalytic properties that may interfere with enzymatic tests, such as those that evaluate endotoxin contamination. These many interferences necessitate the use of inhibition and enhancement controls. These are control samples with known properties included in an *in vitro* assay along with analyte samples to ensure accurate results. For example, in tests to evaluate endotoxin contamination, known amounts of endotoxin can be spiked into nanoparticle samples. If the endotoxin assay reliably measures the true (i.e., known) amount then the researcher can be reasonably sure that the nanoparticles are not interfering with the test method. However, if the test returns a measurement substantially higher (enhancement) or lower (inhibition) than the true amount in the control samples, then the test must be modified before the results can be meaningful.

Figure 1 illustrates the use of inhibition and enhancement controls in a test for endotoxin contamination. Bacterial endotoxin or lipopolysaccharide (LPS) is a membrane component of most bacteria. Administration of a drug contaminated with bacterial endotoxin can cause fever, shock, and even death. Accordingly, the FDA sets limits on the number of endotoxin units (EU), which may be present in a drug or device product. Detection of the products of the *Limulus* amoebocyte lysate (LAL) reaction can be an effective means of quantifying the endotoxin units present in a drug formulation.

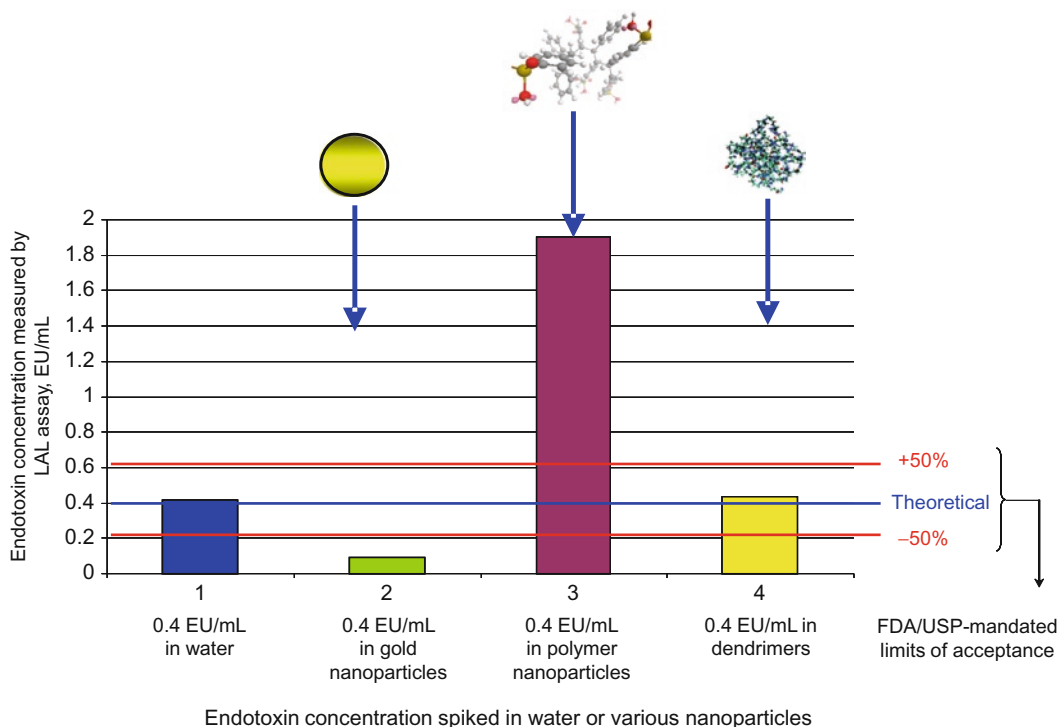


Fig. 1. The line at 0.4 EU/mL represents the assay response which would reflect the true amount of spiked endotoxin in the samples (0.4 EU/mL endotoxin). The lines at  $\pm 50\%$  represent the FDA/USP mandated limits for assay acceptance ( $\pm 50\%$ ). The gold colloid interferes with the assay, the polymer greatly enhances the result, and the dendrimer yields acceptable values.

However, many nanoparticles interfere with the reactivity of endotoxin, the LAL reaction, or the detection of the reaction products. Known amounts of endotoxin can be spiked into samples as inhibition and enhancement controls to evaluate if the particles interfere with the LAL assay. In Fig. 1, water, gold colloid, polymeric, and dendrimeric nanoparticles were spiked with endotoxin inhibition and enhancement controls (a known amount of endotoxin). As can be seen, the LAL test on the water and dendrimeric samples yield a result near the true amount of spiked sample. The gold colloid nanoparticles, however, inhibit the assay, returning an endotoxin reading which is less than the known amount spiked into the sample. The test on the polymeric nanoparticles yields an endotoxin concentration corresponding to a much greater amount of endotoxin than was spiked into the sample. It cannot be concluded from this test alone if the polymeric particles enhance the assay or are in fact contaminated with endotoxin.

Biocompatibility of nanomaterials with blood can be evaluated *in vitro*, and the methods presented in this volume make use of a variety of cell-based *in vitro* systems including immortalized cell

lines or combinations of cell lines and primary cell preparations freshly derived from organ and tissue sources. This volume includes 20 methods for in vitro characterization of nanoparticle samples. These are assays to evaluate sterility, toxicity, and explore immunological properties. Protocols for in vitro assessment of hemolysis, complement activation, and thrombogenicity are presented as these tests are required by the FDA for conventional pharmaceuticals (10). There is also an in vitro test for nanoparticle phagocytosis, which can be predictive of recognition by the immune system and clearance by the reticuloendothelial system (RES).

In summary, the characterization of nanoparticle-based drugs poses a host of novel challenges for scientists, developers, and regulatory agencies. This volume contains protocols for nanoparticle characterization, many of which have been developed at the National Cancer Institute's Nanotechnology Characterization Laboratory (NCL) – an interagency collaboration among NCI, FDA and the National Institute of Standards and Technology (NIST). NCL scientists developed these protocols to rigorously characterize nanoparticle physicochemical properties (e.g., size, aggregation, and surface chemistry), as well as in vitro immunological and cytotoxic characteristics, and ADME/Tox profiles in animal models (Fig. 2). These methods have undergone extensive

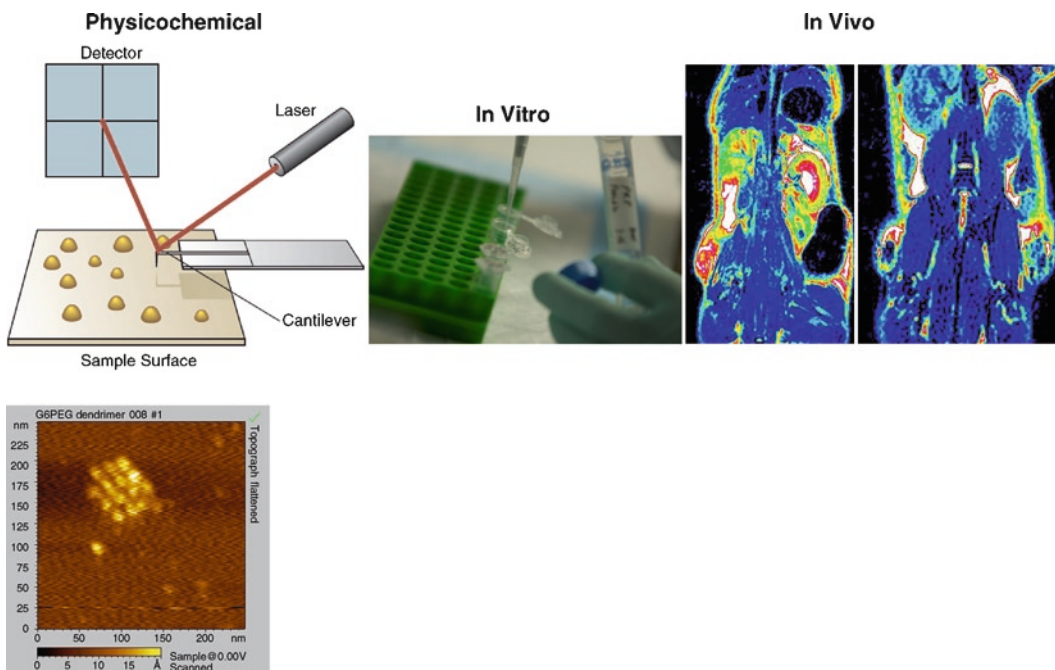


Fig. 2. Characterizing nanoparticles in the NCL assay cascade. Nanotechnology strategies submitted to NCL are characterized in a standardized assay cascade developed in collaboration with the National Institute of Standards and Technology and the Food and Drug Administration. This three-tiered system for nanoparticle characterization consists of physicochemical, in vitro, and in vivo testing.

in-house validation and are subjected to regular revision to ensure applicability to a variety of nanomaterial types. Standardized protocols specific to nanoparticles, such as those presented in this volume, can be employed along with appropriate controls to avoid and overcome many of the challenges described in this chapter.

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