1 Characterization of Nanomaterials in Nanotoxicological Analyses

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1.1 Introduction

In accordance with the European Commission's Recommendation, "Nanomaterial" is defined as a natural, incidental, or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions are in the size range 1 – 100 nm [1]. Nanomaterials (NMs) have attracted great attention because of their unique physical, chemical, and mechanical properties that differ from those of bulk solids and molecules, which enabled them to be widely used in the fields of electronics, chemical industry, medicine, machinery, energy, and so on. With the widespread applications of NMs, the environmental and health impacts of these materials have caused the attention of scientific community, regulatory agencies, environmentalists, industry representatives, and the public. They all agree that more efforts are required to ensure the responsible and safe development of new nanotechnologies. Characterization of NMs is a key aspect in this effort because physicochemical properties of NMs are important factors determining their biological effects and environmental fate. However, there is no universal agreement upon the minimum set of characteristics, although certain common properties are included in most recommendations. Particle characterization is an essential aspect of any attempt to assess potential biological effects of nanoparticulate systems. The thorough characterization of NMs is a daunting task, especially in the context of a complex biological environment. The characteristics of NMs should be measured under conditions as close to the point of application as possible. For toxicology studies, this should include, if possible, the biological environment. For example, if *in vitro* cell studies are being conducted, the particle size should be measured in cell culture media or at least under the same pH and ionic strength conditions.

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Physicochemical properties are the basis for understanding the biological effects of test materials. In this chapter, we emphasize and illustrate the major characterization parameters, including size and size distribution, shape, agglomeration state, crystal structure, chemical composition, surface area, surface chemistry, and surface charge, which should be investigated before, during, and after administration. In addition, the available analytical techniques, methods, and procedures are evaluated to be capable of detecting and quantifying NMs during *in vivo*/*in vitro* studies. These topics provide a comprehensive review of more adequate characterization techniques, methods, and procedures.

1.2 Size and Morphology of NMs

1.2.1 Transmission Electron Microscopy (TEM)

TEM has become one of the most powerful characterization tools in NM research, which provides direct images and information such as the size, shape, morphology, agglomeration state, and crystalline structure of particles at a spatial resolution down to the level of atomic dimensions (<1 nm) [2]. In the conventional TEM mode, an incident electron beam is transmitted through a very thin foil specimen, during which the incident electrons interacting with specimen are transformed to unscattered electrons, elastically scattered electrons, or inelastically scattered electrons [3]. The magnification of TEM is mainly determined by the ratio of the distance between objective lens and the specimen and the distance between objective lens and its image plane. The scattered or unscattered electrons are focused by a series of electromagnetic lenses and then projected on a screen to generate an electron diffraction (ED), amplitude-contrast image, a phase-contrast image, or a shadow image of varying darkness according to the density of unscattered electrons [3]. In addition to the high spatial resolution of TEM, one should ensure that enough particles are examined to provide statistically valid representation of the full size or shape distribution. This can be very difficult and time-consuming and may require the image analysis of literally thousands of individual particles. There are many commercial automated image analysis systems and computer software packages that are used for this purpose. Although TEM is a useful characterization tool, a wide variety of analytical techniques can be coupled with TEM for different applications; for example, energy-dispersive spectroscopy (EDS), electron diffraction (ED), or electron energy-loss spectroscopy (EELS) may be useful for determining additional characterization parameters such as chemical composition and speciation at the atomic scale.

However, there are certain drawbacks accompanying the advantages of TEM. Since a high vacuum and thin sample section are required for electron-beam penetration in TEM measurement, care should be taken to validate the system used against standardized materials and sample preparation [4]. The

representativeness of the sample depends on their dispersion, so it is necessary to select the appropriate disperse conditions to achieve a uniform dispersion of the particles. It should also be noted that electron microscopy normally provides only two-dimensional images, so care must be taken to avoid bias introduced by orientation effects. High-resolution microscopy is subject to artifacts caused by sample preparation or special analysis conditions.

1.2.2
Scanning Electron Microscopy (SEM)

SEM is a surface imaging method in which the incident electron beam scans across the sample surface and interacts with the sample to generate signals reflecting the topographic detail of the specimen surface [4, 5]. The incident electrons cause emissions of elastic scattering of electrons, referring to backscattered electrons, low-energy secondary electrons, and cathodoluminescence from the atoms on the sample surface or near-surface material. Among these emissions, detection of the secondary electrons is the most common mode in SEM and can achieve resolution smaller than 1 nm [5]. It does not require electron-beam penetration in SEM measurement, so it can be used for bulk samples, except for soft biological tissues, which contain large amounts of water.

The size, size distribution, and shape of NMs can be directly acquired from SEM. For conducting materials, the sample preparation is simple, with the size and weight of samples being required for different SEM sample rooms. While for many biological samples with poor electrical conductivity or even insulator, the surface of specimens should be coated by spraying an ultrathin layer of electrically conducting material, such as gold, silver, or other precious metals [4]. When the size of the particles was below 10 nm, the sample cannot be sprayed by gold, for the size of this coating is about 8 nm. The carbon evaporation coating is an alternative method. In short, the samples for SEM should be dry and conductive, as well as the surface structure should be well preserved without deformation or contamination.

1.2.3
Scanning Tunneling Microscopy (STM)

Scanning probe microscopy (SPM) techniques adapt a generic principle, that is, bringing a susceptible probe in close proximity to the surface of an object measured to monitor the reactions of the probe [6]. As the earliest developed technique in the SPM family, STM uses quantum tunneling current to generate electron density images for conductive or semiconductive surfaces and biomolecules attached on conductive substrates at the atomic scale [7–9]. The essential components of STM include a sharp scanning tip, an xyz piezo scanner controlling the lateral and vertical movements of the tip, a coarse control unit positioning the tip close to the sample within the tunneling range, a vibration isolation stage, and feedback regulation electronics. As the tip-sample separation

is maintained in the range of 4–7 Å, a small voltage applied between the scanning tip and the surface causes tunneling of electrons by which variation of the responding current can be recorded while the tip moves across the sample in the *x-y* plane to generate a map of charge density. Alternatively, keeping the responding current unchanged by adjusting the tip height through the use of feedback electronics can generate an image of tip topography across the sample [10].

STM can provide the information of the sample surface at atomic-scale resolution, with parallel resolution of 0.1 nm and perpendicular resolution of 0.01 nm, by using a very sharp tip [11]. It can directly observe the morphology, defects, adsorption, and reconstruction on the surface of specimen by monitoring the structure of single atomic layer on the surface. Different from the samples being usually embedded into a matrix to preserve their original conformations and detection in vacuum in EM techniques, SPM does not need special sample preparation and can perform under environmental conditions, even in water and other solution, with no damage to the sample. Although the high spatial resolution of STM should benefit the characterization of nanoscale biomaterials such as size, shape, structure, and states of dispersion and aggregation, only few studies using gold or carbon as substrates have been reported [12]. The requirements of the conductive surface of the sample and detection of the surface electronic structure were the main practical obstacles, for most biomaterials are insulating and a simple connection of the sample's surface electronic structure with its surface topography may not necessarily exist. Still, STM is a preferred tool for investigating conductive atomic structures of, for example, carbon nanotubes, fullerenes, and graphene [12].

1.2.4 Atomic Force Microscopy (AFM)

AFM does not require electrically conductive surfaces and is a SPM imaging tool consisting of a micromachined cantilever (typically made of silicon or silicon nitride) with a sharp tip at one end to detect the deflection of the cantilever tip caused by electrostatic and van der Waals repulsion, as well as attraction between atoms at the tip and on the measured surface [13–15]. The oscillating cantilever then scans over the surface of specimen to generate an image. Unlike SEM and TEM techniques, which have only high lateral resolutions, AFM can also be used for investigating the size, shape, structure, sorption, dispersion, and aggregation of NMs with a high vertical resolution of around 0.5 nm [16, 17]. The different scanning modes employed in AFM studies include noncontact mode (also called *static mode*), contact mode, and intermittent sample contact mode (also called *dynamic mode* and *tapping mode*). In addition to probing the sizes and shapes of NMs under physiological conditions, AFM is capable of characterizing dynamics between NMs in biological situations, such as observing the interaction of NMs with supported lipid bilayers in real time, which is not achievable with the current EM techniques [18].

The main strength of AFM is its capability to image a variety of biomaterials at the subnanometer scale in aqueous fluids without causing appreciable damage to

many types of native surfaces [19]. However, a major drawback is that the size of the cantilever tip is generally larger than the dimensions of the NMs examined, leading to incorrect estimation of the lateral dimensions of the samples [16]. On the other hand, AFM lacks the capability of detecting or locating specific molecules. Recently, this disadvantage has been eliminated by the progress in single-molecule force spectroscopy with an AFM cantilever tip carrying a ligand, a cell adhesion molecule, or chemical groups, which can probe or detect single functional molecules on cell surfaces [20, 21].

1.2.5 Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS), one of the most popular light scattering techniques, can determine the size distribution of small particles, molecules, or polymers at the scale from submicron down to 1 nm in solution or suspension using a laser as light source [22, 23]. In a DLS experiment, a laser beam is directed at the nanoparticle dispersion, and fluctuations in the intensity of the scattered light are monitored with a photon detector and related to the size of a hypothetical hard sphere that diffuses in the same fashion as the nanoparticles being measured using the Stokes–Einstein equation [23–25]. Thus, the DLS is unsuited to accurately measuring the sizes of nonspherical NMs because equivalent spherical nature of particles is already assumed in the analysis. The polydispersity index (PDI) can indicate the size distribution of the NP dispersion. The larger the PDI, the broader is the size distribution, and a PDI value from 0.1 to 0.25 implies a narrow size distribution [26]. For physicochemical characterization of NMs, the main strengths of DLS include its noninvasive manner, short experiment duration, accuracy in determining the hydrodynamic size of monodisperse samples, and capabilities of measuring diluted samples, analyzing samples in a wide range of concentrations and detecting small amounts of higher molecular weight species, along with lower apparatus costs and more reproducible measurement than other methods [27, 28].

However, it should be noted that DLS measurements can be performed only for a certain range of nanoparticle concentrations, since it is difficult to correlate size fractions with a particular composition when certain amounts of aggregates are present. Also, the nanoparticle suspensions should be sufficiently stable so that there is no significant sedimentation for the duration of the experiment, and dust particles can interfere in the scattering intensity. In addition, DLS has limited utility for analysis of samples with heterogeneous size distributions and resolving the dimensions of a mixed sample population varying in size less than a factor of 3 [27, 29, 30].

1.2.6 X-ray Diffraction (XRD)

An ideal nanocrystalline sample is composed of highly uniform NMs in size and shape. One technique that characterizes both size and crystallinity of NMs at the

atomic scale is X-ray diffraction (XRD). Nanocrystals diffract X-rays in unique ways. The diffraction of X-ray can be simply described as the reflection of a collimated beam of X-rays incident on the crystalline planes of an examined specimen according to Bragg's law. An XRD pattern does not exist in an amorphous sample. Typically, XRD is a tool for characterizing crystalline size, shape, and lattice distortion by long-range order of a crystalline sample. Sizes will change depending on the chemical composition of the crystal. The following Equation 1.1 can be used to determine the grain size of a nanocrystal:

$$D = \frac{0.89\lambda}{\beta \cos \theta} \tag{1.1}$$

where D is the crystallite size, λ is the X-ray wavelength (1.54 Å for Cu Kα radiation), and θ is the Bragg angle. β is taken as the full width at half max of a sample.

Although XRD can confirm a crystalline product and has frequently been used to determine the material structure at the atomic scale, difficulty in growing crystals and the requirement of the amount of powder sample (about 100 mg) limit the applications of XRD technique [31]. Because nanocrystals have such small grain sizes, a long collection time is needed for the sample of interest. In addition, the measured value is more accurate when the grain size is less than 50 nm than the larger ones. Another disadvantage of XRD is the low intensity of diffracted X-rays, particularly for low atomic number materials, compared with ED. A recent X-ray diffraction study reported a new approach using femtosecond pulses from a hard X-ray free-electron laser for structure determination, which may benefit structure determination of macromolecules that do not yield sufficient crystal size for using conventional radiation sources or are not sensitive to radiation damage [32].

1.2.7 Small-Angle X-ray Scattering (SAXS)

Different from XRD, whose applications are limited to crystalline materials, small-angle X-ray scattering (SAXS) provides information of several characteristics by examining either crystalline or amorphous materials in the range of 1 – 300 nm [23, 33, 34]. SAXS can be used to analyze inorganic and organic materials from polymers, proteins to nanoparticles, without special sample preparation process. In SAXS, a portion of an incident X-ray beam elastically scattered from the sample forms a scattering pattern on a two-dimensional flat X-ray detector perpendicular to the direction of the incident X-ray beam. By analyzing the intensity of the scattered X-ray collected within the scattering angle, ranging from 0.1° to 3°, SAXS can evaluate the size/size distribution, shape, orientation, and structure of a variety of polymers and NM-bioconjugate systems in solutions [35].

The features of small-angle scattering in SAXS lead to the capability of studying nonrepeating structures; thus, perfect crystallized structures are not required, which simplifies sample preparation and makes SAXS a nondestructive method. On the other hand, SAXS measurements provide holistic information about the structure of large number of samples, which exhibits the averaged

characteristics rather than local probes of individual grains [34]. This feature can be a disadvantage if high resolution is required. Recently, synchrotron radiation as a high-energy X-ray source has greatly enhanced the resolution of SAXS in the analysis of polymers, proteins, aggregates, gel, catalyst, as well as NMs with different dispersions [36]. However, SAXS is not suitable to measure the particles whose shapes are not such spherical or the mixed powder, which are composed of different materials. In addition, it is important to choose the proper angle for measurement, because the interference effects may impact the accuracy of results.

1.2.8
Brunauer–Emmett–Teller (BET)

Specific surface area, the total surface area of the materials per unit mass (m^2/g), is one of the most important physical properties of NMs, which is usually used to evaluate the ability of reactivity, adsorption, and catalysis of them [37, 38]. Some NMs are designed into porous for the catalyst and adsorption agent. The sizes, shape, volume, and size distribution of pores are very important for these materials [39]. Surface area and porosity of NMs are frequently analyzed using the Brunauer–Emmett–Teller (BET) gas absorption/desorption method [40–42].

The method is based on a model of multilayer adsorption, which satisfies several conditions that adsorption occurs on the adsorbing sites and on top of the adsorbed molecules, that the number of adsorbing sites in each layer is constant, that the energy of the first-layer adsorbing sites is uniform, and that molecules in all layers above the first behave as if in a bulk liquid [39]. Given these conditions, the particle diameter can be calculated according to the specific surface area S_ω per unit weight of the powder by the Equation 1.2:

$$D = \frac{k}{\rho S_\omega} \tag{1.2}$$

where D is the average diameter of the particles, ρ is the density, and k is a factor of particle shape, with different values for different shapes and 6 for spherical particles. It should be noted that this method is only applicable for the spherical particles without pores. On the other hand, BET surface area measurements involve heating of the sample in vacuum before the measurement of nitrogen adsorption/desorption. Thus, the obtained surface area value may not necessarily be a relevant measure of available surface area in a liquid medium due to the fact that NMs in liquid may aggregate together.

1.2.9
Raman Scattering (RS)

Raman scattering (RS) is a widely used tool for structural characterization of NMs and nanostructures that provides submicron spatial resolution for light-transparent material without the requirement of sample preparation, making it suitable for *in situ* experiments [43]. The process of RS records frequency

differences between the incident photons and the inelastically scattered photons associated with the characteristics of the molecular vibrational states, during which the inelastically scattered photons emitting frequencies lower than the incident photons refer to the Stokes lines in Raman spectrum and the inelastically scattered photons emitting frequencies higher than the incident photons are named anti-Stokes lines. The principle of RS is to measure the inelastic scattering of photons possessing different frequencies from the incident light after interacting with electric dipoles of the molecule. RS is generally considered to be complementary to IR spectroscopy, since vibrational modes that are Raman active should be IR inactive, and vice versa. Raman transitions result from nuclear motion modulating the polarizability of the molecules, rather than a net change in the dipole moment of the molecules. Raman spectroscopy can be used to investigate conformations and concentrations of tissue constituents, which demonstrates the potential of RS for detecting tissue abnormalities [44]. Also, it can be used to calculate the average particle size of nanocrystalline by the Equation 1.3:

$$d = 2\pi \left(\frac{B}{\Delta\omega} \right)^{1/2} \tag{1.3}$$

where B is a constant, $\Delta\omega$ is the peak shift for the microcrystalline as compared to the amorphous-like samples [45].

RS is suitable for studying biological samples in aqueous solution because water molecules tend to be weak Raman scatterers. However, while the conventional RS technique provides indirect characterization of NMs, such as average size and size distribution through analysis of the spectral line broadening and shift, it lacks the spatial resolution necessary to delineate different domains for application in nanotechnology [43, 46]. Other disadvantages of conventional RS include interference of fluorescence and extremely small cross section, demanding intense laser excitation and a large amount of sample materials to provide sufficient RS signals [47]. In contrast, implementation of surface-enhanced Raman scattering (SERS) can strongly enhance RS signals and increase spatial resolution while the measured biomolecules are adhered to the surface of metallic structures, such as commonly used Au or Ag NP colloid substrates [48–50]. SERS can be used to (i) study surface functionalization of metallic NPs, (ii) monitor the conformational change in proteins conjugated to the metallic NPs, and (iii) track intracellular drug release from the nanoplatform and measurement of the pH in the surrounding medium [51–55].

1.3 Composition and Structure

In addition to size and shape, chemical composition is another important factor in determining toxicity of NMs. Composition of an NM affects its transport, delivery, and biodistribution. For example, cytotoxicity is generally observed in

quantum dots with core metalloid complexes consisting of widely used metals such as cadmium and selenium [56]. Still, quantum dots can be rendered non-toxic, when core and coatings are appropriately designed [57–59]; alternatively, the cytotoxicity of quantum dots was only observed after degradation of their core coating *in vivo* or *in vitro* [60, 61]. There are several studies addressing toxicological concerns about NPs of different compositions [56]. In biomedical applications of NMs, there may be a need to combine two or more types of NMs to forma complex such as a chelate, a conjugant, or a capsule. Consequently, chemical composition analysis of the NM complex is more complicated than that for a single entity [18].

The atomic absorption spectroscopy (AAS), optical emission spectroscopy (OES), mass spectrometry (MS), energy spectrum analysis, nuclear magnetic resonance (NMR), and X-ray absorption fine structure (XAFS) can be used to analyze the chemical composition or structure of NMs. Some of these methods, such as AAS, OES, and inductively coupled plasma–mass spectrometry (ICP-MS), require performing acid digestion of the samples before determination [42]. Other methods, such as X-ray fluorescence and diffraction analysis, called *non-destructive methods*, allow direct measurement of the solid samples.

1.3.1
Absorption and Emission Spectroscopy

Spectroscopy is one of the most common analytical techniques in nanoscience. This characterization technique measures and interprets various areas of the electromagnetic spectra from either the emission or the absorption of energy by different materials. Different types of spectroscopic techniques can be used to characterize engineered NMs. For example, Raman spectroscopy is used to study vibrational, rotational, and other low-frequency modes in a system and can be utilized to determine the type and degree of functionalization on the sidewall of a carbon nanotube. Absorption spectroscopy is used to quantify the amount of photons a substance absorbs and can be utilized to measure the size of gold nanoshells. Fluorescence spectroscopy is used to analyze the different frequencies of light emitted by a substance, which is then used to determine the structure of the vibrational levels of that substance.

1.3.2
Mass Spectrometry (MS)

MS is one of the major analytical techniques used to examine the mass, elemental composition, and chemical structure of a particle or molecule. The basic principle of MS is to distinguish charged particles with different masses based on their mass-to-charge ratios. MS provides a high degree of precision and accuracy for molecular weight determination, as well as high detection sensitivity, which only requires a small amount of sample (10^{-9} to 10^{-21} mol). Several physicochemical characteristics of NMs, including mass, composition, and structure, can be

depicted using various MS procedures, distinguished by their ion sources, separation methods, and detector systems. Among the ionization techniques coupled with MS analyzers, inductively coupled plasma (ICP) ionization is mainly implemented in the analysis of metal-containing NMs [16, 62]. Applications of different MS procedures for NMs include using time of flight (TOF)-MS to determine the size/size distribution of NMs [63], MALDI-TOF-MS to measure the molecular weights of macromolecules, polymers, and dendrimers as well as to illustrate proteins binding to NMs [18, 64], ICP-MS to validate the conjugation reaction of a functionalized NP with a modified contrast agent [65], and secondary ion MS to access the elemental and molecular properties of the top layer of NPs, as well as to examine biomaterial surface properties in physiological conditions [66, 67]. Although these MS techniques have been applied to the analysis of physicochemical properties of various biomolecules, the currently incomplete MS spectral databases still cause difficulties in identifying molecular species, for example, in the analysis of MALDI-TOF-MS outcome measures [68]. Additionally, the applications of MS techniques for NMs to date are constrained in NM-bioconjugate characterization, mainly due to the cost of instrumentation, sample destruction, and necessary instruments generally supplied for other investigations [23].

1.3.3 X-ray Fluorescence Spectrometry (XRF)

X-ray fluorescence spectrometry (XRF) can be used to measure the solid samples, which has great advantages in the analysis of chemical composition of NMs. X-rays are electromagnetic radiation and are manifested in two forms, continuous radiation and characteristic radiation. Continuous radiation is produced when a high-energy electron beam decelerates as it approaches the electron clouds that surround the atomic nucleus. Characteristic radiation is produced following the ejection of an inner orbital electron by high-energy particles and subsequent transition of atomic orbital electrons from states of high to low energy. There is a simple relationship between the emission wavelengths and the atomic number of the excited element. XRF, in which primary X-rays are used to excite characteristic secondary radiation from the specimen being analyzed, is a technique used for qualitative and quantitative elemental analysis [69, 70].

The sample for XRF analysis can be a solid or a solution. Powdered samples of NMs can be pressed into discs or be directly placed in the sample cell and then be measured. Analysis of NM suspensions can be performed by dropping the liquids on the filter paper and drying them. Thin-film samples of NMs can be directly determined. The advantages of composition analyses of NMs using XRF are listed as follows: (i) a wide range of elements, from ^{4}Be to ^{92}U, can be measured; (ii) the spectral lines of X-ray fluorescence are plain with less interference; (iii) the analysis method is simple and noninvasive; and (iv) a wide range of concentrations, from constant to trace, of elements can be analyzed.

1.3.4
Nuclear Magnetic Resonance (NMR)

In contrast to imaging and diffraction techniques affording structural information at long-range order, that is, the crystalline property, NMR is sensitive to the local environment to resolve the structures of amorphous materials, polymers, and biomolecules that lack long-range order. In addition to evaluating the structures and compositions of the species, NMR spectroscopy can be used to investigate dynamic interactions of the species under different conditions [16, 23]. The relaxation, molecular conformation, and molecular mobility can be evaluated through different dynamic measurements using specifically designed RF and/or gradient pulse sequences [71]. NMR spectroscopy has been implemented to determine several physiochemical characteristics of NMs, including structure, purity, and functionality in dendrimers, polymers, and fullerene derivatives, as well as conformational changes occurring in the interactions between ligands and NMs [72–75]. Pulsed field gradient NMR has been implemented to evaluate the diffusivity of NMs, through which the sizes and interactions of species under investigation can be calculated [76].

NMR is a nondestructive technique that requires little sample preparation. However, the low detection sensitivity of NMR, in contrast to optical techniques, requires a relatively large amount of the sample for measurement. It can also be time-consuming if a certain level of signal-to-noise ratio is necessary for spectral analysis.

1.3.5
X-ray Absorption Spectroscopy (XAS)

X-ray absorption spectroscopy (XAS) is an effective method to study the composition and structure of NMs. It includes X-ray absorption near-edge structure spectra (XANES) and extended X-ray absorption fine structure (EXAFS). XANES can yield information about chemical state and symmetries of the absorption site of the specific atom. EXAFS, the oscillatory structure in the X-ray absorption coefficient, contains much of the local structure near an absorbing atom without the requirement of long-range order in the measured species, including quantitative information concerning coordination numbers, near-neighbor distances, and structural and vibrational disorder in bond distances [31, 77]. EXAFS can provide the averaged structural information of a NM, resulting from a local order of samples examined in the manner of inelastic X-ray interaction with the samples. Because of the high energy and broad spectrum of the synchrotron radiation source, EXAFS has become one of the most effective methods to study the structure of the specific atom in the material. It can provide the local structure information near the absorption atom at about 0.002 nm.

XAS has enabled scientists to analyze samples that could not be analyzed using classical techniques such as XRD and proven to be a powerful technique in several fields including biology, and environmental and material sciences. In

addition, it allows for the direct determination of elemental oxidation states and local coordination environments of specific elements, where the use of other methods is time-consuming, is cumbersome, and can lead to false results [78, 79]. Detailed description of XAS and its applications in nanotoxicology studies can also be found in Chapter 5 of this book.

1.4 Surface Properties

The surface properties of NMs are expected to contribute substantially to the mode and extent of their biological effects. Surface composition, energy, charge, and reactivity clearly affect NM interactions with biomolecules and biological systems. It is recommended that an interactive approach to surface characterization be undertaken, since it is often impractical to characterize the full spectrum of surface properties for each NM. It is also suggested that NMs are stored under inert conditions and the surface composition and structure of the "as-received" NMs should be measured at the very minimum. Where possible, measurements should also be performed on the NMs "postexposure."

1.4.1 Surface Area

There has been a good deal of discussion regarding the role of surface area in NM toxicology. Some researchers have argued that surface area plays an important role in the toxicity of NMs and is the measurement metric that best correlates with particle-induced adverse health effects [1, 80, 81]. There is a growing consensus that the potential for adverse health effects is most directly proportional to particle surface area. Since surface area almost always scales with size (at least for nonporous materials), one could equally make the case that size is the best correlation. In fact, if one measures the mass, density, and primary particle size distribution of a material, the approximate surface area can be readily calculated by assuming spherical geometry. Thus, the question is not so much what surface area of material was used to dose an animal or cell culture, but rather whether the dose should be normalized by total surface area concentration rather than mass or number concentrations.

Particle surface area is clearly an important characteristic of an NM and should always be measured. As described previously, the surface area of NMs can be accurately measured using gas adsorption and the BET method. For particles dispersed in water or air, measuring surface area is somewhat more difficult. Aqueous dispersions can be carefully dried and the resulting powders measured by BET. Care must be exercised to ensure that the surface area is not perturbed by drying and that there are no other salts or components of the solution that influence the measurement.

1.4.2 Surface Charge

The surface charge may reflect the native NM surface or the adsorption of ions and biomolecules at their interface, which will influence the dispersion stability of NMs in aqueous solutions. In an ionic solution, the surface of a charged particle is firmly bound to opposite charged ions, forming a thin liquid layer named Stern layer, which is encompassed by an outer diffuse layer consisting of loosely associated ions. These two layers compose the so-called *electrical double layer* [82]. Zeta potential refers to the sign and magnitude of charge at the shear plane, which divides the fluid envelope that associates itself with the particle and the bulk solution phase. It is usually determined by measuring the velocity of the charged species toward the electrode in the presence of an external electric or acoustic field across the sample solution [23, 25].

Zeta potentials of NMs are typically measured by electrophoresis or electroacoustic methods. The light-scattering electrophoresis is the most commonly used technique since commercial fully automatic instruments are widely available in the market [83]. The equipment is designed to measure the movement of the NMs under an applied electrical field by laser Doppler velocimetry (LDV). To obtain reliable results, the material of interest must have a refractive index sufficiently different from that of the dispersing medium and the dispersing medium must be transparent to light. The absolute value of zeta potential greater than 30 mV indicates a stable condition, whereas a low zeta potential value of less than 30 mV indicates a condition toward instability, aggregation, coagulation, or flocculation [23]. Zeta potential can be significantly affected by the concentration of the sample, pH, temperature, and ionic composition of the dispersion medium [82, 84, 85]. When reporting the value, these parameters should be clearly specified. In particular, pH should be measured just before or after in the dispersion in which the zeta potential was measured, but not in the solution used for preparing the dispersion [86]. The particles will adjust their surface charge and zeta potential according to the current solution composition. The choice of sample concentration is particle-specific and is dependent on the particle scattering properties. High concentrations will lead to multiple scattering and significant particle interactions and hence will result in experimental artifacts [82]. On the other hand, a precise, repeatable zeta potential measurement in a diluted solution cannot reflect the true value in a concentrated suspension [84]. In addition, the values of zeta potential will change with the temperature since the viscosity of the dispersing media depends on the temperature, although the effect is not dramatic [87]. Therefore, zeta measurement should be carried out with temperature control and the results should be reported with the temperature. One should also pay attention to the ionic strength and composition of the dispersing medium. Ions have the potential to adsorb on the particle surface and thus affect the surface charge distribution. Surface complexes or coatings can also be formed and lead to a change in zeta potential. Air bubbles introduced to the solution during sample filling should

also be avoided. Any obstacle along the optical path would have an effect on light scattering and result in experimental artifacts or irreproducible data.

1.4.3
Surface Composition

NMs possess a very high surface-area-to-volume ratio, and the molecular composition and structure of the surface will ultimately define their chemistry. However, it is often difficult to directly measure the atomic composition of surfaces because many of these systems are subject to trace surface contaminants that may not be detectable by general chemical analysis. Directly measuring the atomic composition of “as-received” or “as-administered” NMs is very important. Postexposure examination of changes in surface composition and structure will undoubtedly provide priceless clues with respect to their behavior and the fate of these NMs in biological systems. Methods such as X-ray photoelectron spectroscopy (XPS) and secondary ion massspectroscopy (SIMS) have been extensively used for characterizing NMs, as well as correlating biomaterial surface properties to physiological endpoints [67]. Many of the methods used for surface characterization require ultrahigh-vacuum environments ($<10^{-5}$ Pa). Under such conditions, the surface properties and bonding structure of some materials have been shown to change. Therefore, washing and remova1 of biomolecules from the surface of the particles are likely to be a necessary step. Care must be taken to prevent artifacts from this process.

XPS, also known as *electron spectroscopy for chemical analysis* (ESCA), is one of the most widely used techniques for analyzing the surface composition of NMs. The popularity of XPS comes from its ability to identify and quantify the elemental composition of the outer 10 nm or less of any solid surface for all elements from lithium to uranium on the assumption that the element of interest exists at greater than 0.05 atomic%. Each element has a characteristic electronic structure and thus a characteristic XPS spectrum. Since the binding energies of the electron orbitals in atoms are known, the positions of the peaks in the XPS spectrum can be used to identify the atomic surface composition of the sample. XPS not only allows the identification of the elements constituting the sample but also provides information on their oxidation state based on the binding energy or chemical shift. For example, it has been used for the estimation of the concentration of oxygen vacancies in CeO_2 NPs, based on the concentration of Ce^{3+} and Ce^{4+} [88]. However, it should be noted that the use of XPS to estimate the concentration of oxygen vacancy defects on the basis of oxygen concentration or the fitting of the oxygen peak in the XPS could result in erroneous estimates due to the presence of oxygen in any molecules on the surface of the samples.

Auger electron spectroscopy (AES) is another common surface analysis tool for NMs. Both AES and XPS detect electrons emitted from samples with kinetic energies typically below 2000 eV. In AES, the sample is irradiated with electrons instead of X-rays [89]. AES and XPS provide similar information, but AES gives a higher lateral resolution since the electron beam can be focused to a smaller size than

X-rays. However, the electron beam can also impart more damage to the sample surface than X-rays. Other ion-based techniques, such as time-of-flight secondary ion mass spectrometry (TOF-SIMS) and low-energy ion scattering (LEIS), can provide information on surface coatings, functional groups, or contaminants to complement the electron spectroscopic techniques.

1.4.4
Surface Reactivity

Some NMs actively participate in oxidation or reduction reactions when interacting with biological systems. The surface reactivity of NMs can be measured through comparative microcalorimetry, via the use of probe molecules that are monitored for either degradation or changes in oxidative state, or through a number of electrochemical methods. All of these techniques can potentially be used to monitor particle reactivity in biological fluids, although losses in sensitivity and artifacts are likely. The choice of method will depend on the types of molecular transformations that occur at the particle surface. Comparisons between the surface compositions of the "as-received" particles and those exposed to biological systems can provide further insight for selection.

The surface energy and wettability of NMs can be important for understanding NM aggregation, dissolution, and bioaccumulation behavior. The surface energy of NM systems can be measured through heat of immersion microcalorimetry studies or through contact angle measurements with various liquids. Dynamic and static contact angle measurements can be performed to determine directly the particle wettability within biological fluids. Phospholipids, proteins, and other biomolecules are known to adsorb to surfaces in physiological fluids and change their wettability and sometimes biodistribution characteristics [90, 91].

1.5
Interactions between NMs and Biological Environments

With the increasing applications, NMs can be released into the environment intentionally or accidentally. When NMs are introduced into the environment or biological systems, many undesirable effects such as aggregation, coagulation, and nonspecific absorption can occur. These may be due to a variety of intermolecular interactions occurring at the interfaces of NMs with biomolecules and interaction-mediating fluids [92]. The toxic effects will depend not only on the initial properties of NMs, including chemical composition, shape, surface geometry and crystallinity, porosity, heterogeneity, and hydrolytic stability, but also on the physicochemical evolution in the surroundings, such as ionic strength, pH, temperature, and the presence of biological or organic macromolecules, which can characterize the surface charge, dissolution, hydration, size distribution, dispersion stability, agglomeration, and aggregation of NMs in a given medium [93–96]. Thus, it is necessary to assess the physicochemical characteristics of

NMs before use, at the moment of delivery, as well as after exposure to the *in vitro* or *in vivo* test [97]. Techniques for determining the shelf life of NM formulations are essential before considering the manufacture. For example, it is important to guard against degradation of the NMs caused by moisture, oxidation, and/or aggregation. In this respect, different characterization techniques will be useful for quality assurance.

1.6 Conclusions

Given the novelty of physicochemical characteristics at the nanometer scale, NMs have potential to impact interactions in the biological environment from the molecular to the systemic level. The rapid development and production of NMs for use indicate the demand for comprehensive determination of the properties of NMs. Robust techniques for characterization of NMs are fundamental to regulatory guidelines for ensuring safety of NMs in general use and toxicological studies. This chapter introduces different methods that are commonly used for characterizing NMs. Indeed, it is necessary to characterize the NM in both its originally manufactured condition and after introduction into a physiological environment. These issues become quite complex when trying to determine the mechanism of toxicity. Outside of the body, the environmental conditions can be manipulated to promote the dispersion and stability for measuring size and surface chemistry, while in the biological environment, one is restricted to the conditions under which the organism lives. Often, properties can only be measured after NMs are removed or tissues fixed, potentially introducing artifacts into the measurements. Until better techniques for characterization in these environments are developed, researchers must use the tools available to reconstruct the particle properties *in situ* and how they interact with biological systems. The brief description of each technique, together with its strengths and limitations, provides us with a picture for selecting suitable techniques for characterization of NMs.

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