

Chapter 2

Characterization of Nanomaterials for Toxicological Studies

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Abstract

The scientific community, regulatory agencies, environmentalists, and most industry representatives all agree that more effort is required to ensure the responsible and safe development of new nanotechnologies. Characterizing nanomaterials is a key aspect in this effort. There is no universally agreed upon minimum set of characteristics although certain common properties are included in most recommendations. Therefore, characterization becomes more like a puzzle put together with various measurements rather than a single straightforward analytical measurement. In this chapter, we emphasize and illustrate the important elements of nanoparticle characterization with a systematic approach to physicochemical characterization. We start with an overview describing the properties that are most significant to toxicological testing along with suggested methods for characterizing an as-received nanomaterial and then specifically address the measurement of size, surface properties, and imaging.

Key words: Particle characterization, Size distribution, Surface charge, Surface chemistry, Microscopy

1. Introduction

The increased emphasis on nanotechnology and its potential widespread commercial applications over the last two decades has sparked great interest in the potential human health and environmental effects of nanomaterials. Most of this attention is directed toward particulate nanomaterials as these are the most likely to be spread through the environment as aerosols or water-borne suspensions. If uncontrolled and unregulated, there is some potential in the minds of many that such particles can pose a human health hazard and/or environmental damage. Although the history of nanotechnology may trace its roots back to 1959 with Richard Feynman's "There is room at the Bottom" essay and popularized by Alex Drexler's 1991 book *Unbounding the Future: the Nanotechnology Revolution*, the first serious attention to the potential toxic effects of nanotechnology came much later (1, 2).

In the spring of 2003, several groups presented findings at the annual American Chemical Society meeting illustrating cases where the size of nanomaterials affected their distribution and toxic behavior in animal studies (3). This brought national and international attention to the issue. The next year, the Royal Society published a report, *Nanoscience and nanotechnologies: opportunities and uncertainties*, that focused serious attention on the health and safety implications of nanotechnologies (4). In the Fall of 2004, the University of Florida, in conjunction with the National Toxicology Program, held the first US National Symposium on experimental approaches available to evaluate the toxicity of nanomaterials (5). Since that time, numerous workshops, conferences, organizations, and regulatory guidelines have been initiated to address this pressing issue. The journal, *Nanotoxicology*, was created in 2007 and the Society of Toxicology now has a specialty section focusing on nanotoxicity. Because of the uncertainty in the health and safety effects of nanotechnology, funding for nanotoxicity research has gradually increased with a US National Nanotechnology Initiative FY2011 (all agency) budgetary request of \$117 million devoted to EH&S research (out of the 1.76 billion total request). Virtually all researchers in the field acknowledge the importance—and the difficulty—of sound characterization of nanomaterials as a necessary element in assessing their toxicity and/or other biological activity (6–9).

Particle characterization is an essential aspect of any attempt to assess potential biological effects of nanoparticulate systems. This may seem obvious, but to those unaccustomed to analyzing or quantifying particulate systems, the thorough characterization of their nanomaterials is a daunting task, especially in the context of a complex biological environment. Most traditional particle size analyzers have been designed to measure particle properties under controlled conditions with limited confounding factors (10, 11). In mineral or chemical systems, one often has the luxury of adjusting the environment by dilution, changing the solvent system, adjusting the pH, or adding surfactants to promote dispersion and aid the analysis. In biological systems, the presence of multiple components, high ionic strength, a limited temperature range, and potential toxic effects of dispersion aids hamper the investigator in his ability to measure important particle attributes. Properties such as size distribution, state of aggregation, surface charge, surface chemistry, translocation, and interaction with biological components can be very difficult to quantify and interpret in these complex environments (12). In the end, characterization becomes more like a puzzle put together with various measurements rather than a single straightforward analytical measurement. In this chapter we emphasize and illustrate the important elements of nanoparticle characterization with a systematic approach to physicochemical characterization. Subheading 2 is an overview describing the

properties that are most significant to toxicological testing along with suggested methods for characterizing an as-received nanomaterial. Subheadings 3–5 address the measurement of size, surface properties, and imaging, respectively.

2. Measurements and Methodologies

A number of individual researchers and several National and International organizations have pooled resources to try and define which properties of nanomaterials are needed to best evaluate or predict their toxicological behavior (6, 8, 13, 14). Most researchers agree on the basic characterization parameters. These usually include those shown in Table 1.

There are many other properties that have been suggested for the complete characterization of Nanomaterials. Perhaps the most

Table 1
Important properties for characterization of nanomaterials

As-received	In vitro ^a	In vivo
Particle size distribution	Stability	ADME
Particle shape	Surface chemistry	Translocation/distribution
Bulk composition	Zeta potential	Agglomeration
Purity	Cytotoxicity	Immune response
Solubility	Hemolytic properties	Inflammation
Surface chemistry ^b	Surface adsorbed species	Toxicity
Surface area	ROS generation	
Surface morphology (crystallinity, shape, surface roughness)	Sterility	
Hydrophobicity		
Zeta potential		
Stability/agglomeration behavior		

^aSee NIST-NCL protocols at <http://ncl.cancer.gov/>

^bSurface chemistry is often rather broadly defined (if defined at all). Most often it is meant to describe the chemical species on the surface, their concentration, and their effect on the interaction with the surrounding solvent system, surfaces, and other suspended solids. See Subheading 2 for greater detail

complete list has been compiled by OECD's Working Group on Manufactured Nanoparticles (15). This list includes a number of additional properties such as dustiness, water-octanol partition coefficient, a TEM micrograph, and number of other surface properties. Ultimately, it is up to the individual researcher to decide which properties are pertinent to his or her experiment. For example, the OECD list includes "dustiness" which may be important in the setting of aerosol exposure limits but may be irrelevant to an *in vitro* or parenteral exposure.

Once the properties of interest are identified, there are two issues that must be addressed: measuring the properties and expressing the measurement in some standard fashion acceptable to other scientists. The "dustiness" property is a good example because it is somewhat obscure in its definition (16). Intuitively, we may all understand that "dustiness" relates to the propensity for a dry powder to become aerosolized. However, there are different methodologies for quantifying this property oriented toward specific industries, size ranges, and concentrations (e.g., ASTM Standard D 7486-08). A dustiness measure designed for the coal industry likely will not adequately capture this property for dry agglomerated nanomaterials. Such a measurement for dry nanomaterial applications has yet to be standardized.

Fortunately, if we go back to the list in Table 1, we will find that for most (but not all) of these measurements there are established analytical methods (17). Perhaps the most notable exception is the "agglomeration behavior" of particulate systems. The quantification of this important property is a major issue as particles are introduced into biological environments and will be treated in more detail in the next section.

The bulk chemical composition and purity of the material is usually assessed by standard analytical means. For metals and metal oxides, the elemental composition can be measured by inductively coupled plasma (ICP-AES or ICP-MS), atomic absorption, or other quantitative techniques. Impurities can also be assessed by these means. For polymers or other organic constituents standard analytical techniques can be applied, sometimes requiring dissolution in appropriate solvents (18). A variety of methods such as Gel permeation chromatography, FTIR, NMR, LCMS, and others can be used to determine the structure and average MW of polymers. Analytical techniques can be highly specialized and often require sophisticated instrumentation and special expertise. If the nanomaterial is procured commercially, one of the most obvious sources of this information is the manufacturer. Manufacturer's technical support personnel are usually willing to provide additional information that may not be on the specification sheet for their materials. It is always useful, however, to conduct one's own analysis if possible, as the manufacturer may not quantify the same information that is relevant for toxicological studies. For example, a few

ppm of copper or heavy metals in silver nanoparticles may not materially affect the intended antimicrobial properties of the silver but, if concentrated near the surface, may have a dramatic effect on its toxicity toward higher organisms. We once found 0.50 ppm mercury in a 50 ppm colloidal silver preparation that was intended for human consumption! The method of manufacture, transport, dispersion, storage, or even atmospheric exposure can sufficiently alter the surface composition of some nanomaterials so as to impart toxicological properties significantly different from the bulk material. Hence purity should always be a concern.

Solubility is often overlooked as being relatively straightforward. When it comes to toxicity, it is not. Looking up the “solubility” in a CRC handbook and ascribing “soluble” or “not soluble” is often insufficient to characterize the solubility of nanomaterials. Almost all metals and metal oxides have some solubility in aqueous systems and even very slight solubilities can have significant metabolic/toxic effects. Amorphous silica has an equilibrium solubility of 90 ppm at pH 7 which increases dramatically as particle diameter decreases below 10 nm (19). The previous example of silver nanoparticles is another case in point. The equilibrium solubility of silver in deionized water is low enough (only a few ppm) that it would generally be described as insoluble, yet its solubility is high enough to exert a deadly effect on microbes. Solubility can change dramatically with particle size due to increased surface energy, and changes in pH can have very dramatic effects on the dissolution and solubility of particles. Many metals are multivalent and amphoteric, and the hydrolysis and speciation of metal cations can be an important parameter (e.g., Cr^{3+} vs Cr^{6+}). At the very least, the equilibrium concentration of soluble constituents should be established and the aqueous chemistry of the nanomaterial should be well understood when conducting toxicological tests (20).

3. Size, Size Distribution, and Agglomeration

The determination of particle size seems very intuitive and simple to the layman but is far more challenging and complex than is generally recognized. Both ISO and ASTM have committees and numerous working groups devoted exclusively to particle characterization and particle size measurement (ISO TC24, SC4 particle characterization, and ASTM E-29 on particle and spray characterization). The complexity in particle characterization lies in two basic attributes: particles are generally small and hence they are generally numerous. Representative sampling is a crucial (often overlooked) issue. A large number of particles must be sampled and measured to adequately represent the size distribution, especially for broad size distributions. The second attribute is that

particles have a natural tendency to agglomerate in solution or “coagulate” from an aerosol dispersion (21). Unless stabilized by some form of repulsive surface forces (typically electrostatic or steric), van der Waals forces will cause particles to stick together as they move in Brownian motion and randomly come into contact with each other. Hence, one is often dealing with a dynamic system where the particle (or at least agglomerate) size changes as a function of time or with changes in the surrounding environment.

Most individual particle sizing techniques do not clearly differentiate between primary particles and particle agglomerates. In addition, most measurement techniques interpret or report particle size based on the assumption that the particles are spheres with homogeneous properties (e.g., uniform density, refractive index). When agglomeration causes particle shape or density to depart from these assumptions, the assessment of the particle size distribution becomes less reliable. Thus, multiple analysis techniques and imaging are normally combined to develop a more complete picture of size distribution and state of dispersion. An example of this is provided in Figs. 1, 2, and 3 for a nominally spherical 50 nm aluminum sample. In this example the particle size was determined by three common methods: electron microscopy, BET surface area, and laser diffraction. Primary particle size appears to be qualitatively distributed in the 20–100 nm range by TEM/SEM. The aluminum powder has a specific surface area of 35.1 m²/g and a median diameter by laser diffraction as recorded below in Table 2.

Figure 1 shows the laser diffraction size measurement of the Al powder dispersed and sonicated in deionized water. Note how different the laser diffraction data looks when plotted as a number distribution versus the volume weighted distribution. The number distribution is calculated to be the relative frequency of particles of a given diameter while the volume distribution is weighted by the volume or “cube” of the particle diameter. A particle system with a broad size range or with a high degree of agglomeration will be evident by a greater disparity between the two distributions when

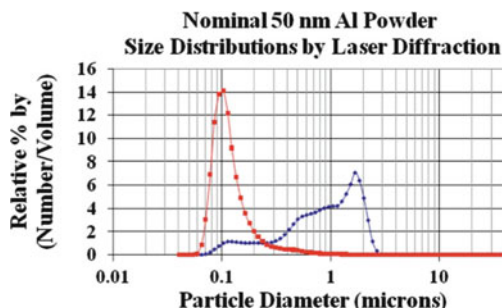


Fig. 1. Coulter LS13320 laser diffraction particle size for a nominal 50 nm aluminum sample (number and volume distributions).

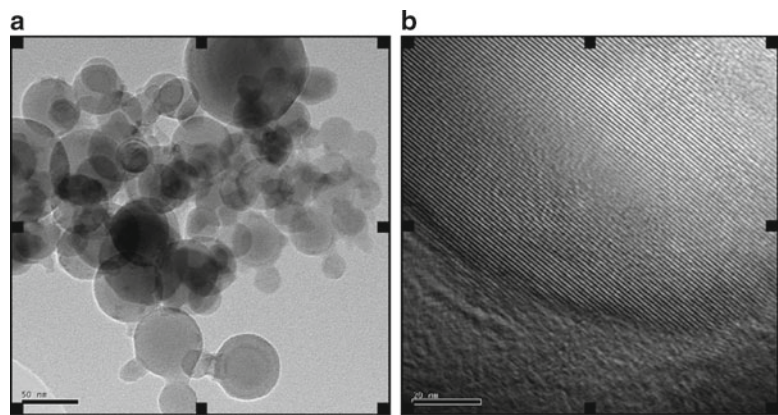


Fig. 2. TEM micrographs of aluminum 50 nm sample. (a) Aggregated particles, (b) high mag showing crystalline FCC lattice in particle interior and amorphous surface coating. Scale bars = 50 nm (a) and 20 nm (b).

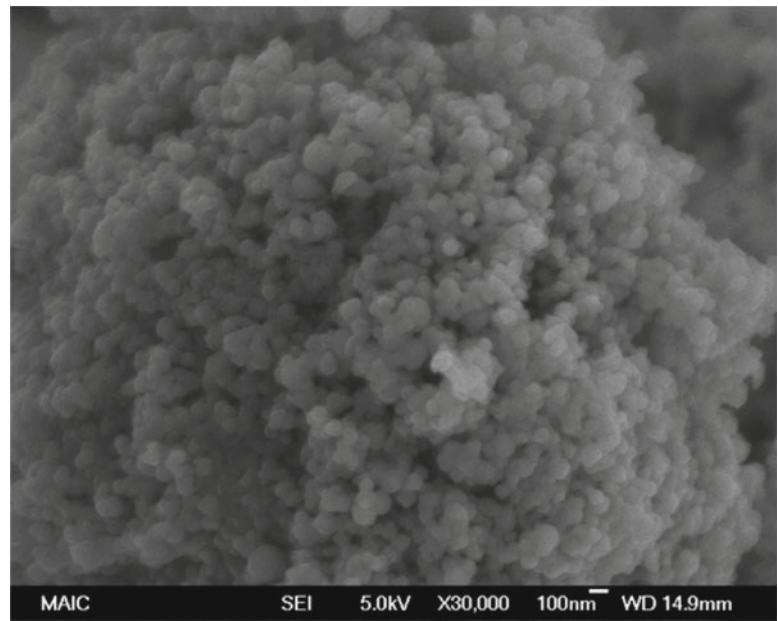


Fig. 3. SEM micrographs of as-received aluminum 50 nm powder.

Table 2
Particle size data for a nominally 50 nm Al powder

50 nm Al powder

Median diameter (number)	111 nm
Median diameter (volume)	1.02 μm
Specific surface area	35.1 m^2/g (~56.9 nm sphere)

they are plotted side by side. In this example, the additional information gained by BET surface area analysis and by SEM/TEM (Figs. 2 and 3) enables one to conclude that the primary particle size distribution lies predominantly between 20 and 100 nm, and that the powder is substantially agglomerated when dispersed in water.

In assessing particle size distribution for toxicity testing there are additional issues to be addressed. Since aggregate size is often dynamic, there is the question of when and under what conditions to make the measurements. Usually particles are characterized most thoroughly *as-received* from the manufacturer. Here the researcher has the maximum control over the environment and may freely manipulate the system to measure the desired properties. For example, the pH of the solution may be adjusted to promote dispersion, surfactants may be used, sonication or high shear can be applied to promote dispersion, and particles can be prepared for microscopy with fewer potential artifacts. Most measurement techniques are not designed for complex systems; therefore the best results are usually attained by keeping the particle-solvent system as simple as possible. Ensemble techniques usually measure properties averaged over a large number of particles in the measurement zone (e.g., dynamic light scattering (DLS), laser diffraction). Thus, assuming that the sampling is representative, the results are statistically robust. Once the properties of the material are thoroughly understood *ex vivo*, samples can be prepared appropriately for exposure with greater confidence and understanding.

For nanomaterials, the most common wet sizing techniques are DLS, laser diffraction, centrifugal sedimentation, acoustic techniques, Brownian motion analysis, electrozone sensing, and dark field, fluorescent, or confocal microscopy, albeit optical microscopy is limited to viewing relatively large nanoparticle agglomerates. For very small nanoparticles, large macromolecules, dendrimers and proteins, size exclusion chromatography (SEC), asymmetric field flow fractionation (AFFF), and ultracentrifugation are popular methods. For dry powders or aerosolized nanomaterials one can determine particle size from BET-specific surface area, dynamic mobility analysis (DMA), time of flight mass spectroscopy (TOFMS), light scattering, and electron microscopy. Atomic force microscopy (AFM) and cantilever resonance techniques (e.g., quartz balances, microfluidic cantilevers) can be conducted on either dry or wet samples.

All of these measurement techniques become more difficult to implement in complex heterogeneous systems such as cell culture media and biological fluids. The complexity increases in three ways. Additional components can influence the aggregation state of the particles (often by a change in pH, ionic strength, or surface adsorption). Secondly, they influence important physical properties of the surrounding medium such as viscosity, refractive index, or

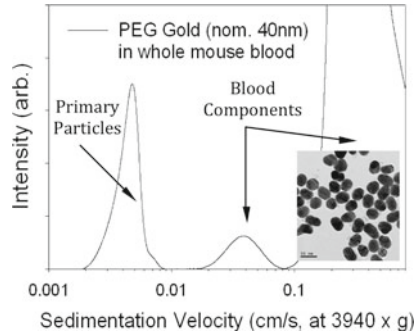


Fig. 4. Analytical centrifugation data for PEG-coated 40 nm gold in whole blood. *Inset*: TEM micrograph of primary particles (50 nm bar).

absorbance, and third, additional moieties in biological systems are often particles themselves (e.g., large proteins, biopolymers, cells, microparticles, etc.) that can confound or obscure the measurement. In Fig. 4, centrifugal analysis demonstrates these phenomena for a sample of PEG-coated 40 nm gold particles dispersed into whole blood. There appears a relatively distinct peak (at the equivalent of 40 nm) representing the primary gold particles with two peaks representing various cellular components of the lysed blood. The cellular components of the blood present a background which can easily obscure larger gold aggregates present in the sample. In this example, the analysis is further complicated by the fact that the various components have distinctly different densities.

Due to its broad popularity, DLS deserves a few comments of its own. DLS is based on the principle that particles diffuse under Brownian motion as a function of their hydrodynamic diameter (or size), fluid viscosity, and temperature. Smaller particles tend to diffuse more rapidly than larger particles. Mathematically, the diffusion of a particle in a fluid is described by the Stokes–Einstein equation. Again, in this basic equation the particle is assumed to be spherical and homogeneous.

$$d_h = \frac{k_B T}{3\pi\eta D_T},$$

where d_h = hydrodynamic diameter, k_B = Boltzman constant, T = temperature (K), η = solvent viscosity, and D_T = translational diffusion coefficient. At a given temperature and viscosity, the hydrodynamic diameter can be calculated by experimentally determining the diffusion coefficient.

In DLS, the diffusion coefficient is usually derived through the use of photocorrelation or power spectrum analysis (Doppler shift). Without delving into the mathematical details, one can see that for uniform, well-dispersed, homogenous spheres or emulsions, there will be a single diffusion coefficient, and DLS is highly reliable and

robust (22). Various advanced algorithms enable DLS to be used for polydisperse samples, but the accuracy suffers the further a sample deviates from the ideal. As with most other techniques, highly aggregated, contaminated, or heterogeneous samples can yield poor results if the researcher does not use appropriate care in making the measurement. Though not strictly defined as DLS, Brownian motion analysis can be applied to individual particles if their individual movement is captured by video with a dark field microscope and laser illumination. The motion of individual particles is captured and analyzed to determine their diffusion coefficient, and a statistical picture of the whole sample is developed by the automated analysis of many particles. This technique suffers from its own weaknesses including high sensitivity to sample concentration, clarity and depth of field, and a relatively smaller sample size. However, as a counting technique, it can handle multimodal or broad distributions.

The preceding discussion highlights the care that must be taken when conducting particle size analysis before, during, and after exposure in toxicological studies. Multiple techniques are almost always essential to develop a full understanding of primary particle size and state of agglomeration. The investigator should thoroughly understand the physical principles by which these measurements are made and give careful consideration to the composition of the surrounding fluid and how it affects these measurements. Collaboration with researchers trained in particle characterization is always a good idea.

4. Surface and Interfacial Properties

Particles are all about size, shape, and surface properties. The most obvious surface property for fine particles is the high surface area. For spheres, specific surface area increases inversely with particle diameter, thus for a given mass it increases dramatically as particle size approaches the nano range. The theoretical specific surface area is usually measured in meters squared per gram. For a spherical particle it can be calculated by the following formula where density (ρ) is described in g/cm³ and diameter is given in microns.

$$SSA = \frac{6}{\rho \times d_{\text{microns}}}.$$

Thus, a 1 μm silica sphere ($\rho=2.2 \text{ g/cm}^3$) has a SSA of 2.72 m²/g, whereas a 10 nm silica particle has a 100-fold greater area of 272 m²/g! A perfect sphere is the shape of minimum surface area, thus any shape deviation, surface roughness, or porosity serve to increase the specific surface area. This equation is most often

used to calculate the spherical equivalent particle size from a BET surface area measurement (23). The BET measurement should be included in the particle characterization whenever there is sufficient dry powder available. For wet systems, or where there is insufficient material available to conduct BET analysis, surface area is most often approximated by measuring particle size and calculating the surface area using a spherical assumption (24). Occasionally, surface area is approximated in solution by dye adsorption or acid/base titration. For example, the Sears test is a standard acid base titration method used for determining surface area in the colloidal silica industry (25). There has been some success in measuring the in situ surface area of aerosol powders using the epiphaniometer and more recently by a diffusion charging (DC) technique (26, 27).

Surface area is important because it is the surface that interacts with the surroundings; however, it is not surface area alone that defines these interactions. Surface chemistry consists of the chemical structure of particle surfaces, development of surface charge, surface functional groups, surface active sites, and the propensity to adsorb moieties from the environment both pre- and postexposure. Surface adsorption and/or coating can completely change the nature of the surface. Surface chemical analysis is most easily conducted on as-received materials. Two principle measurements of surface chemistry are hydrophilicity and zeta potential. These will largely determine how the particles physically respond to dispersion in aqueous fluids. The two are related in that hydrophobic particles seldom develop a substantial surface charge and therefore may be difficult or impossible to disperse in solution. They may however have a greater affinity for lipids, membranes, or other lyophilic environments.

There is no easy way to quantify the contact angle of particles. Typically researchers will simulate the particle surface composition on a flat bulk surface and measure contact angle accepting the fact that this is an approximation of the highly curved nanoparticle surface. Another method is by powder capillary rise using the Laplace–Washburn equation; however, this technique suffers from insufficient precision in defining the effective interparticle pore radius (r_{eff}) for a nonspherical packed nanoparticle bed plus difficulty dealing with hysteresis effects (28).

$$\Delta P = \frac{2\gamma LV \cos \theta c}{\gamma_{\text{eff}}}.$$

One will note that the OECD working group on manufactured nanomaterials suggests using the octanol–water partition coefficient to characterize hydrophilicity (15). This method is used routinely in the pharmaceutical industry and by environmental toxicologists to partition and identify hydrophobic species dissolved in aqueous solutions. Its usefulness for nanoparticles is still

under debate. In its most simplified form, nanoparticles are suspended in water mixed with an organic solvent, and the concentration of nanoparticles that partition into each phase is quantified through a partition coefficient ($\log P$). This parameter can be used in a comparative manner against standard materials to estimate the hydrophobicity of a powder (29).

$$\log P = \log \left(\frac{C_o}{C_w} \right).$$

There are several issues with this simplified technique. It fails to account for effects such as particle size, sedimentation, and surface tension effects at the water–octanol interface. Standardized methods for applying it to nanoparticles are still under consideration.

A more quantitative method of determining the hydrophilicity of particles is by AFM. In this method, particles are affixed to an AFM cantilever and force measurements are recorded as the AFM tip is lowered into a drop of water (30, 31). This is a rather specialized technique with its own set of issues such as how particles are affixed to the cantilever tip and their geometry as the tip approaches the water droplet. It has not yet been adapted as a standardized method but is very useful if the instrumentation and expertise are available.

Since almost all exposures in toxicology studies eventually involve aqueous biological fluids, it is important to determine the surface charge that develops on particles when introduced into aqueous solutions. Since the charge at the particle surface is difficult to measure directly, the zeta potential (net potential at the shear plane) and isoelectric point of the particles are typically used to quantify particle charge (32). A complete analysis should be made in both deionized water and in a buffered solution of similar ionic strength and pH relevant to the intended exposure route. Figure 5 shows a zeta potential titration of aluminum nanoparticles and demonstrates the sensitivity to the increase ionic strength of tap water and the adsorption of even small concentrations of polyvalent ions. When measuring (or attempting to measure) zeta potential, the dispersion stability of the particles in suspension often becomes quite evident and provides insight into the agglomeration behavior of the materials. For aqueous suspensions, zeta potential is generally positive below the isoelectric point (low pH) and negative above the IEP. One should be alert that as pH approaches very high or very low values solubility often increases dramatically.

Generally, zeta potentials of absolute value greater than about 30 mV are characteristic of well-stabilized colloids. As the pH approaches the isoelectric point, dispersions become less stable and more prone to agglomeration. Due to the high ionic strength and near neutral pH of many physiological fluids, it is common for nanoparticle systems to become totally unstable and agglomerate

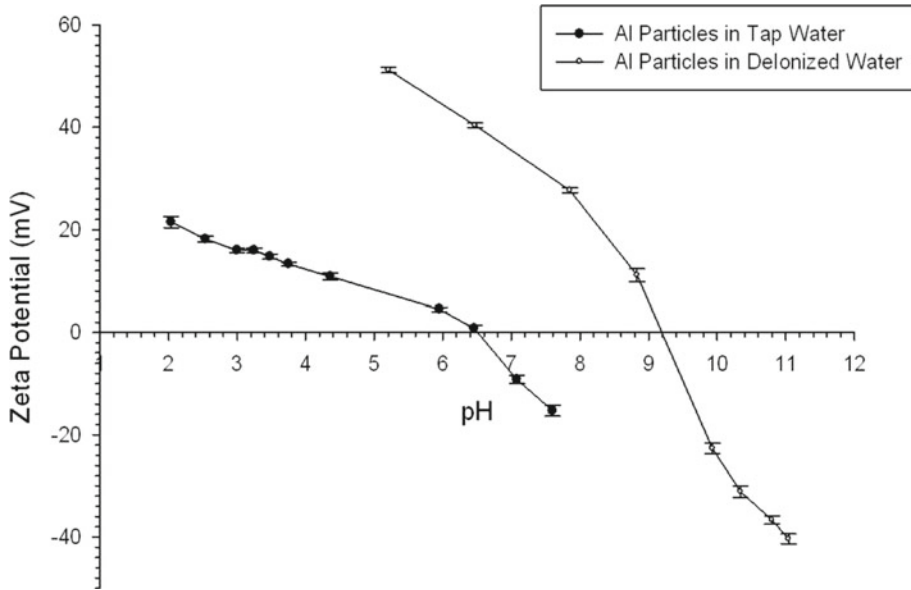


Fig. 5. Zeta potential titration of aluminum nanoparticles in both deionized and tap waters.

rapidly when introduced into the biological environment. The temptation at this point is to attempt to disperse the particles through the use of surfactants or other dispersion aids, high energy sonication, shear, coatings, other surface modifications, or by changing the solution conditions. The danger is that such treatments may promote unrealistic exposure protocols and begs the question, “Are the biological effects due to the particles or to the surface modifications used to disperse them?”

Zeta potential can be measured with several good techniques including electroacoustic, DLS, and electrophoretic methods (33–35).

The functional surface chemistry of nanomaterials consists of a broad range of interfacial properties that, like zeta potential, rely on the surroundings as well as the particle surface. Thus, surface composition and properties should be characterized as-received with the understanding that these properties may change once introduced into a biological setting. The elemental composition of the particle surface can be determined by surface sensitive techniques such as X-ray photoelectron spectroscopy (XPS), secondary ion mass spectroscopy (SIMS), energy dispersive spectroscopy (EDS), and auger electron spectroscopy (AES). These are all high vacuum techniques and vary in their capabilities and surface sensitivity. Surface chemistry may also change substantially when particles are handled, dried, fixed in a biological matrix, and placed under ultra-high vacuum for analysis. Molecular species can sometimes be deduced from these analyses (e.g., bond energies from XPS), but painting a complete picture of the surface atomic layers

on particles is still an art. Standard spectroscopic techniques such as FTIR and Raman can be helpful in identifying surface groups, but suffer from issues such as sensitivity and (too high) depth of penetration. Fortunately, unless a specific surface chemistry is intentionally introduced, surface groups tend to be some combination of the bulk particle composition, oxides, hydroxyl groups, or contaminants introduced during synthesis or handling. Surface treatments, such as a peroxide baths (Fenton treatment) or plasma cleaning, can be used to clean surfaces of impurities with the understanding that one may be imparting an artificially “clean” surface to the particles prior to exposure (36).

The propensity for nanomaterials to catalyze or generate free radicals or reactive oxygen species is often of specific interest in assessing toxicity. Many semiconductor or transition metal oxides such as iron oxides, titanium dioxide, and cerium oxide can be catalytic toward free radical species under different conditions (37). Fullerenes, carbon nanotubes, graphene, along with various functional groups can also generate radicals (38, 39). While the mechanisms of ROS generation may or may not be a specific goal of the investigation, quantification of ROS generation is important in the toxicological evaluation of nanomaterials. Quantification of ROS in solution or in vitro is often accomplished using various colorimetric or fluorometric indicators such as dihydrorhodamine-123 (DHR) which is converted to the fluorescent form upon reaction with peroxide, singlet oxygen, and other reactive species (40, 41). Electron paramagnetic resonance (EPR) or spin resonance (ESR) is a specialized method that can be used to measure free radicals directly or by “spin trapping” radicals with a reporter molecule. It is a powerful in vitro technique and has seen increasing applications in in vivo detection and quantification of oxygen and ROS (42).

Clearly there are many other reactions that take place at the surface of particles in the biological environment. There may be specific uptake of proteins, specific cell membrane interactions, opsonization of particles, and numerous other interactions all of which might bear on the toxicity profile of these materials. Particles designed for therapeutic or imaging applications have specific surface properties engineered to chemically target them to the desired location or to control the lifetime in circulation. Consequently, the surface chemistry of nanoparticles can be as complex and as broad as the field of analytical chemistry itself.

5. Microscopy and Imaging

Microscopy is often considered the gold standard for measuring the properties of nanoparticles because it is visual, more intuitive, can be highly quantitative, and there is less ambiguity in the

observed size, structure, and morphology (6). It is generally recognized as good practice to include micrographs at the appropriate magnification of any nanomaterial being characterized for toxicity testing. For nanomaterials, this normally will require scanning electron microscopy (SEM), transmission electron microscopy (TEM), or AFM. These techniques can contribute toward the measurement of the particle size, shape, morphology, particle size range, crystallinity, agglomeration state, purity, surface area, and surface morphology. The chemical composition of the nanoparticles and coatings can be determined using energy dispersive X-ray spectrometry (EDS) or electron energy loss spectroscopy (EELS). As with all particle characterization techniques, sampling and statistical reliability are two major issues when drawing conclusions from imaging alone. It is much more difficult to achieve and verify a representative sample for the very small samples required for microscopy, and there are many artifacts that are possible in the preparation and analysis of samples especially for in vacuo techniques such as SEM and TEM.

Optical techniques generally don't have the resolution to see individual nanoparticles but are useful to image the cells, tissue, and for pathology. Fluorescent probes can be embedded or surface attached to particles to track their behavior and location. Usually, TEM with the appropriate sample preparation and staining techniques is required to analyze the nanoparticles in the ultrastructure of cells and tissue. Morphological information is gained through comparison of images taken using multiple techniques over a wide range of magnifications (43, 44). For example, Fig. 6 shows a series of optical and TEM images at different magnifications that "home in" on the location, size, and aggregation state of gold nanoparticles deposited from the blood stream in the liver of a mouse. The EDS spectrum is necessary to positively identify the particles, which are often confused with staining artifacts or cellular structures.

A better understanding of the interactions between cells and nanoparticles is obtained using multiple microscopy techniques. Correlative microscopy includes any available microscopic techniques, including light, probe, laser, and electron microscope techniques. A few of the microscopy techniques that are typically used in biological sciences and the cellular uptake of nanoparticles are transmitted light, fluorescence microscopy, confocal microscopy, and TEM with elemental analysis such as EDS or EELS (44–47). The analysis can be in two or three dimensions. Three-dimensional analysis is more difficult to achieve, but can provide additional information on shape, particle distribution, and relationship to various cellular structures. Confocal imaging, stereo SEM, cryo-sectioning, AFM, and soft X-ray tomography are all techniques that provide 3D data. Typically, an optical technique is used to screen samples and to locate areas of interest. TEM is needed for ultrastructure information obtained through high resolution

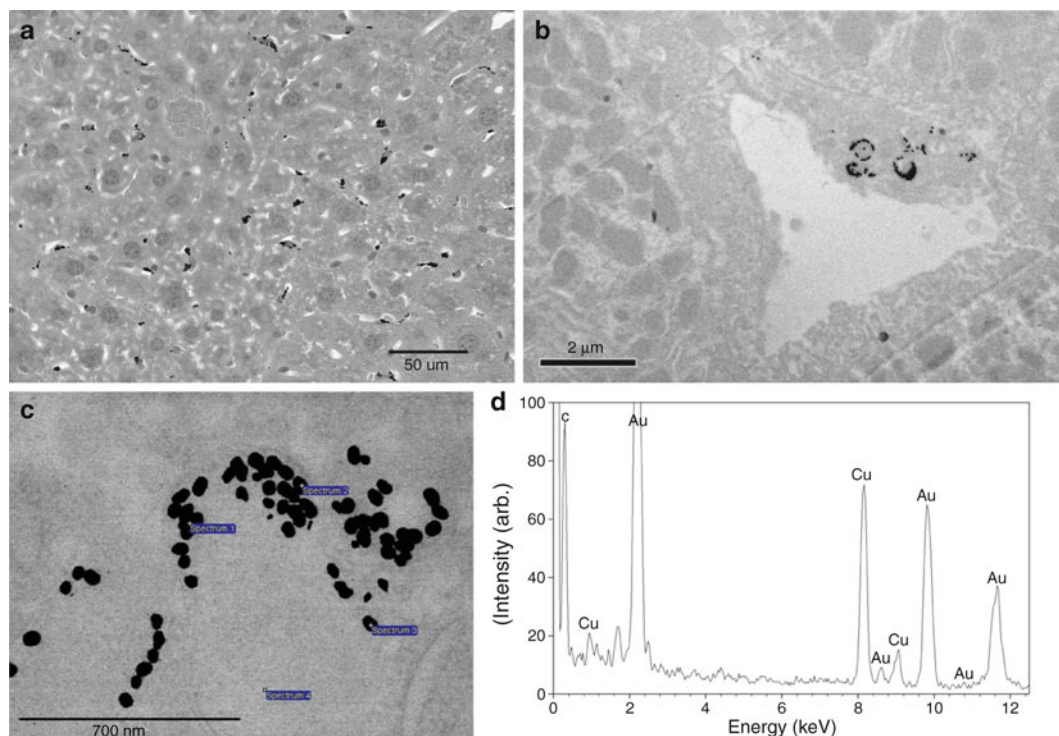


Fig. 6. Optical and TEM images of the gold nanoparticles in the Kupffer cells of a mouse liver with the EDS spectrum confirming elemental composition. (a) Optical micrograph of gold aggregates in a histological liver section. (b) TEM of particles in liver section at higher magnification. (c) TEM at still higher magnification showing primary gold particles. (d) EDS spectra taken by TEM confirming that the particles in the image are gold (copper peaks are from TEM grid). Scale bars = 50 μm (a), 2 μm (b), and 700 nm (c).

imaging and elemental analysis. The preparation of samples for TEM requires special expertise that is expensive and labor intensive. In many cases, the TEM results are necessary to aid in the interpretation of the optical results, after which the optical technique can be used as a screening process.

The latest advances in TEM analysis of biological materials are in cryo preparation, cryo TEM, and TEM tomography (48–50). Cryo preparation procedures, such as high pressure freezing, eliminate artifacts from the conventional preparation procedures of fixation, dehydration, and embedding. The possibility of washing away nanoparticles is also eliminated when high pressure freezing is used. The frozen samples can be imaged directly in the cryo TEM without staining. TEM tomography is based on a tilt series of two-dimensional projections of the object along different directions that is reconstructed into a three-dimensional projection of the original object.

Ion abrasion SEM (IA-SEM) or focused ion beam SEM (FIB-SEM) is being used to present three-dimensional views of cells and tissue (48–53). It is a relatively new technique to the world of

biological sciences. It combines the removal of thin layers of samples by an ion beam and then imaging the newly exposed face of the sample. This slice and image technique combined with stereological image analysis software can be used to build the 3D image from SEM slices. The result is an image cube that shows the nanoparticle distribution in three dimensions.

Some of the more exciting recent advances in biological imaging include X-ray microscopy and soft X-ray tomography. The resolution of X-ray microscopy lies between that of the optical microscope and the electron microscope. X-ray microscopy using synchrotron soft X-ray sources such as the Advanced Light Source at Berkeley Labs has achieved resolutions as fine as 15 nm. The advantage of X-ray microscopy is that biological samples can be analyzed in their natural hydrated state, preserving the unaltered microstructure of the cell. Soft X-ray tomography provides three-dimensional imaging much like the medical computed tomography (CT) scan we are all familiar with, but at much higher resolutions. The biggest drawback to the widespread use of X-ray-based biological imaging has been the need for a synchrotron source of X-rays. However, commercial instruments with laser plasma X-ray sources are now being developed with submicron capabilities and the potential to do small animal imaging (54, 55).

Imaging is currently the only method available to assess the shape of nanoparticles. Although many nanoparticulate systems are nominally equiaxed or spherical, many of the most interesting structures are not. Carbon nanotubes (CNTs), nanowires, and nanoflakes might pose a significant risk if toxicity is linked directly to shape factors. High aspect fibers (e.g., asbestos) have been linked to mesothelioma at larger particle sizes and hence researchers and regulatory agencies are cautious. There have been a number of studies to try and determine if shape is a factor in the toxicity of CNTs and, although there are a number of studies showing pulmonary or cellular toxicity, it is still inconclusive (56, 57). Nonetheless, shape affects many other physical properties such as aerosolization, aggregation, and diffusion and should be quantified in any characterization protocol.

6. Conclusions

There has been a great deal of attention and research devoted to the issue of nanotoxicology over the last 10 years. The scientific community, regulatory agencies, environmentalists, and most industry representatives all agree that more effort is required to ensure the responsible and safe development of new nanotechnologies. Characterizing nanomaterials is a key aspect in this effort. There is no universally agreed upon minimum set of characteristics

although certain common properties are included in most recommendations (see Table 1 and additional references). Reviewers for many professional journals have been sensitized to look for such information. Ultimately it is up to the researcher to decide the properties that are the most pertinent to the goals of the investigation and to characterize their nanomaterials accordingly. Research teams incorporating multidisciplinary personnel can be most helpful in this respect.

References

1. Feynman RP (1959) There's plenty of room at the bottom: an invitation to enter a new field of physics. American Physics Society, Caltech Engineering and Sciences
2. Drexler E, Peterson C, Pergamit G (1991) Unbounding the future: the nanotechnology revolution. William Morrow & Co., New York
3. Service RF (2003) Nanomaterials show signs of toxicity. *Science* 300:243
4. Society R (2004) Royal Society and Royal Academy of Engineering Report on nanoscience and nanotechnologies: opportunities and uncertainties (Society TR, ed), London
5. Bucher J, Masten S, Moudgil B, Powers K, Roberts S, Walker N (2004) Developing experimental approaches for the evaluation of toxicological interactions of nanoscale materials. Final Workshop Report, Gainesville
6. Oberdorster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, Carter J, Karn B, Kreyling W, Lai D, Olin S, Monteiro-Riviere N, Warheit D, Yang H (2005) Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol 2:8
7. Thomas K, Sayre P (2005) Research strategies for safety evaluation of nanomaterials, Part I: evaluating the human health implications of exposure to nanoscale materials. *Toxicol Sci* 87:316–321
8. Powers KW, Brown SC, Krishna VB, Wasdo SC, Moudgil BM, Roberts SM (2006) Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. *Toxicol Sci* 90:296–303
9. Borm PJA, Robbins D, Haubold S, Kuhlbusch T, Fissan H, Donaldson K, Schins R, Stone V, Kreyling W, Lademann J, Krutmann J, Warheit D, Oberdorster E (2006) Part Fibre Toxicol 3:11–46
10. Allen T (ed) (2004) Powder sampling and particle size measurement, vol I, 5th edn. Chapman & Hall, London
11. Allen T (ed) (2004) Surface area and pore size determination, vol 2. Chapman & Hall, London
12. Knapp J, Barber T, Lieberman A (1996) Liquid- and surface-borne particle measurement handbook. Marcel Dekker, New York
13. OECD (2008) OECD Environment, Health and Safety Publications Series on the safety of manufactured nanomaterials No 6, Document ENV/JM/MONO (development, O. f. e. c. a., Ed.), Paris
14. Scientific Committee on Emerging and Newly-Identified Health Risks (SCENIHR) (2007) Opinion on the appropriateness of the risk assessment methodology in accordance with the technical guidance documents for new and existing substances for assessing the risks of nanomaterials, European Commission
15. OECD (2010) Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme; first revision ENV/JM/MONO, (development, O. f. e. c. a., Ed.), Paris
16. Boundy M, Leith D, Polton T (2006) Method to evaluate the dustiness of pharmaceutical powders. *Ann Occup Hyg* 50:453–458
17. Nieman TA, Skoog DA, Holler FJ (2006) Principles of instrumental analysis. Brooks/Cole, Pacific Grove
18. Holler FJ, Skoog DA, West DM (1996) Fundamentals of analytical chemistry. Saunders College Publications, Philadelphia
19. Iler RK (1979) The chemistry of silica. Wiley, New York
20. Baes CEJ, Messmer RE (1976) The hydrolysis of cations. Krieger Publishing Co, Malabar
21. Hinds WC (1999) Aerosol technology: properties, behavior, and measurement of airborne particles. Wiley, New York
22. Berne B, Pecora R (1976) Dynamic light scattering. Wiley, New York
23. Brunauer S, Emmett PH, Teller E (1938) *J Am Chem Soc* 60:309–319
24. Maynard AD (2003) Estimating aerosol surface area from number and mass concentration measurements. *Ann Occup Hyg* 47:123–144

25. Sears GW (1956) Determination of specific surface area of colloidal silica by titration with sodium hydroxide. *Anal Chem* 28: 1981–1983
26. Baltensperger U, Gaggeler HW, Jost DT (1988) *J Aerosol Sci* 19:931–934
27. Jung HJ, Kittelson DB (2005) Characterization of aerosol surface instruments in transition regime. *Aerosol Sci Technol* 39:902–911
28. Forný L, Saleh K, Denoyel R, Pezron I (2010) *Langmuir* 26(4):2333–2338
29. Weisner MR, Bottero JY (eds) (2007) *Environmental nanotechnology: applications and impacts of nanomaterials*. McGraw-Hill, New York
30. Rabinovich YI, Yoon RH (1994) Use of atomic force micro-scope for the measurements of hydrophobic forces. *Colloids Surf A Physicochem Eng Asp* 93:263–273
31. Yakubov GE, Vinogradova OI, Butt HJ (2001) A study of the linear tension effect on the polystyrene microsphere wettability with water. *Colloid J* 63:518–525 (Translated from *Kolloidnyi Zhurnal* 63(4):567–575)
32. Delgado AV, Gonzalez-Caballero F, Hunter RJ, Koopal LK, Lyklema J (2005) Measurement and interpretation of electrokinetic phenomena (IUPAC Technical Report). *Pure Appl Chem* 77:1753–1850
33. Booth F (1948) Theory of electrokinetic effects. *Nature* 161:83–86
34. Dukhin SS, Semenikhin NM (1970) *Koll Zhur* 32:366
35. O'Brien RW, Hunter RJ (1981) *Can J Chem* 59:1878
36. Martin R, Alvaro M, Herance JR, Garcia H (2010) Fenton-treated functionalized diamond nanoparticles as gene delivery system. *ACS Nano* 4:65–74
37. Nel A, Xia T, Madler L, Li N (2006) Toxic potential of materials at the nanolevel. *Science* 311:622–627
38. Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJ (2004) Nanotoxicology. *Occup Environ Med* 61:727–728
39. Garza KM, Soto KF, Murr LE (2008) Cytotoxicity and reactive oxygen species generation from aggregated carbon and carbonaceous nanoparticulate materials. *Int J Nanomed* 3:83–94
40. Brehm M, Schiller E, Zeller WJ (1996) Quantification of reactive oxygen species generated by alveolar macrophages using lucigenin-enhanced chemiluminescence – methodical aspects. *Toxicol Lett* 87:131–138
41. Hanson KM, Clegg RM (2002) Observation and quantification of ultraviolet-induced reactive oxygen species in ex vivo human skin. *Photochem Photobiol* 76:57–63
42. Holley AE, Cheeseman KH (1993) Measuring free radical reactions in vivo. *Br Med Bull* 49:494–505
43. Jahn K, Barton D, Braet F (2007) Correlative fluorescence and Scanning transmission electron microscopy for biological investigation. *Mod Res Educ Top Microsc* 1:203–211
44. Porter AE, Muller K, Skepper J, Midgley P, Welland M (2006) Uptake of C60 by human monocyte macrophages, its localization and implications for toxicity: studied by high resolution electron microscopy and electron tomography. *Acta Biomater* 2:409–419
45. Kapp N, Studer D, Gehr P, Geiser M (2007) Electron energy-loss spectroscopy as a tool for elemental analysis in biological specimens. *Methods Mol Biol* 369:431–447
46. Stearns RC, Paulauskis JD, Godleski JJ (2001) Endocytosis of ultrafine particles by A549 cells. *Am J Respir Cell Mol Biol* 24:108–115
47. Yen HJ, Hsu SH, Tsai CL (2009) Cytotoxicity and immunological response of gold and silver nanoparticles of different sizes. *Small* 5: 1553–1561
48. Robinson JM, Toshihiro T, Pombo A, Cook PR (2001) Correlative fluorescence and electron microscopy on ultrathin cryosections: bridging the resolution gap. *J Histochem Cytochem* 49:803–808
49. Sartori A, Gatz R, Beck F, Rigort A, Baumeister W, Plitzko JM (2007) Correlative microscopy: bridging the gap between fluorescence light microscopy and cryo-electron tomography. *J Struct Biol* 160:135–145
50. van der Wel NN, Fluittsma DM, Dascher CC, Brenner MB, Peters PJ (2005) Subcellular localization of mycobacteria in tissues and detection of lipid antigens in organelles using cryo-techniques for light and electron microscopy. *Curr Opin Microbiol* 8:323–330
51. Heymann JA, Hayles M, Gestmann I, Giannuzzi LA, Lich B, Subramaniam S (2006) Site-specific 3D imaging of cells and tissues with a dual beam microscope. *J Struct Biol* 155:63–73
52. Heymann JA, Shi D, Kim S, Bliss D, Milne JL, Subramaniam S (2009) 3D imaging of mammalian cells with ion-abrasion scanning electron microscopy. *J Struct Biol* 166:1–7
53. Matthijs de Winter DA, Schneijdenberg CTWM, Lebbind MN, Lich B, Verkleij AJ, Drury MR, Humbel BM (2009) Tomography of insulating biological and geological materials using focused ion beam (FIB) sectioning and low kV imaging. *J Microsc* 223: 372–383

54. Yamamoto Y, Shinohara K (2002) Application of X-ray microscopy in analysis of living hydrated cells. *Anat Rec* 269:217–223
55. Parkinson DY, McDermott G, Etkin LD, Le Gros MA, Larabell CA (2008) Quantitative 3-D imaging of eukaryotic cells using soft X-ray tomography. *J Struct Biol* 162:380–386
56. Lam CW, James JT, McCluskey R, Arepalli S, Hunter RL (2006) A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. *Crit Rev Toxicol* 36:189–217
57. Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WA, Seaton A, Stone V, Brown S, Macnee W, Donaldson K (2008) Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nat Nanotechnol* 3:423–428

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