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Review Article

Analytical methods for nanomaterial characterization

P. Khalid[1]
V.B. Suman[2,*]
S. Shameema[2]
N. A. Vinodini[2]
R. Pai. Shela[2]
Pratik K. Chattergee[2]

[1]*Department of Biotechnology, P A College of Engineering, VTU. Mangalore, Karnataka, India.*
[2]*Department of Physiology, Manipal University. Kasturba Medical College. Mangalore, Karnataka, India.*

* Corresponding author:
V.B. Suman
Department of Physiology,
Manipal University.
Kasturba Medical College.
Mangalore, P.O.Box 575001,
Karnataka, India.
Tel +91 824 2211746
Fax +91 824 242 8183
Email suman.vb14@gmail.com

ABSTRACT

In recent years, it has become evident that it is necessary to systematically and accurately define particle characteristics in order to understand the potential toxicity of nanoparticles to biological systems. The properties that need to be emphasized are size, shape, dispersion, doping, aggregation, functionalization, physical and chemical properties, surface area, and surface chemistry. Route of exposure are Oral, dermal, inhalation and injection. Toxicity screening strategy; *In Vitro* Assays, *In Vivo* Assays and cell type are also an important parameter to consider while studying the toxicity of nanoparticles. These analytical methods used to check the toxicity require expensive equipment and sampling, which are time consuming. Using the bacteria, as a biosensor, solves these problems. This method is sensitive, low-cost and easily reproducible and takes 5 to 30 minutes to predict toxicity.
This review focuses to develop a synergy among different paradigm of Nanomaterials characterization.

Keywords: *Nanomaterials; Scanning Electron Microscopy (SEM); Atomic Force Microscopy (AFM); Dynamic light scattering.*

INTRODUCTION

Historically, the commercial growth and application of some potentially useful substances have turned out to have negative health consequences among producers and users if those potential impacts are not acknowledged and investigated early. For example, asbestos was commercially used because of its good insulating and fireproofing properties, but later it was determined to cause significant health problems 20 to 30 years after exposure. Other useful chemicals such as DDT and lead were also determined to be unfavorable to human health and the environment, years after they were put into market. Mass production of chemicals before investigating potential health concerns and societal impacts has significant human burden and financial consequences because of the high cost of remedial actions to remove these hazardous materials and because of the medical and liability costs incurred for compensating individuals with an exposure-related disease or injury.

Nanotechnology is a rapidly emerging material science technology with global economic benefits. Concern over the lack of knowledge about the potential health risks associated with the handling of pure, unbound engineered nanomaterials has been expressed by investors, entrepreneurs, government agencies, and public health advocacy groups. Such concerns create potential barriers to the growth of Nanotechnology and the commercialization of nano enabled products and devices could help address serious global problems concerning energy, transportation, pollution, health, medicine, and food. The potential benefits of nanotechnology are huge; however, these benefits may not be appreciated unless a determined effort is made to evaluate the safety and health concerns regarding these new nanomaterials and to develop effective methods for their responsible introduction.

Therefore, early and accurate characterization of these materials is essential to determine their toxicological effects [1]. Certain characteristics have been identified which must be considered for the characterization of nanoparticles prior to study the toxicity. These properties are size, shape, dispersion, physical and chemical properties, surface area, and surface chemistry [2-4]. A synergy need to be developed between the material science and the toxicological science to understand this complex issue of the nanoparticle toxicity [5]

Categorization of Nanoparticles

- ***Unintentionally produced nanoparticles/ Ultrafine Particles (UFPs)***

The large number of UFPs exists in the atmosphere. Particles in the atmosphere are defined as either primary or secondary particles. Primary particles are emitted directly from sources or processes, which might be natural (fires, volcanoes, sea spray and erosion) or anthropogenic (traffic, industry). Secondary particles are formed in the atmosphere by gas-to-particle conversions. Immediately following nucleation the secondary particles are very small (~1–10 nm), and grow by coagulation or condense onto existing sub-micrometer particles. Homogenous nucleation, the formation of very small particles may occur in hot combustion gases and in metallurgical processes, including welding. Examples, internal combustion engines, Power plants, Incinerators, Jet engines, Metal fumes (smelting, welding, etc.), Polymer fumes, other fumes, Heated surfaces frying, broiling, grilling and Electric motors.

- ***Intentionally produced nanoparticles/ Engineered Nanoparticles (ENPs)***

The U. S. National Nanotechnology Initiative (NNI) define nanoscale materials with properties that are often unique due to their dimensions of 1 to 100 nanometers (nm), and that are intentionally manufactured.

Examples, Carbon-based ENPs include single-walled and multi- walled carbon nanotubes (SWCNTs and MWCNTs), spherical fullerenes and dendrimers. Metal and metal oxide ENPs includes cadmium in various complexes, gallium arsenide, gold, nickel, platinum, silver, aluminum oxide (alumina), cerium dioxide (ceria), silicon dioxide (silica), titanium dioxide (TiO_2, titania), and zinc oxide.

Nanoparticle characterization techniques

It is unanimously agreed that the recommended practices is essential for basic characterization of nanomaterials. These recommended practices should be developed jointly by physical scientists skilled in nano characterization and biological scientists experienced in toxicology research.

Complete characterization of nanoparticles includes size and size distribution, shape and other morphological features (e.g., crystallinity, porosity and surface roughness), chemistry of the material, solubility, surface area, state of dispersion, surface chemistry, and other physicochemical properties.

To some degree, the characterization varies on the objectives of the study. However, there are a number of essential properties that must be measured for test materials used in nanotoxicity studies [2-4]. These include size and shape, state of dispersion, physical and chemical properties, surface area, and surface chemistry. In the following sections, different analytical techniques are discussed which could be used to measure each of these characteristics.

- ***Dynamic light scattering***

Dynamic light scattering (DLS) is also called photon correlation spectroscopy can be used to measure geometrical structure and their state of

motion by scattering light from small particles [9]. DLS is a useful technique to evaluate the particle size, size distribution, and the zeta potential of nanomaterials in solution [2]. Zeta potential is the key factor to determine solution stability [10]. The increase in zeta potential would guarantee the solution stability. DLS technique has been used in the past studies for detection of geometrical structure [6] and is being used in recent time for analyzing the suspension stability and particle size in solution. For example, metal and metal oxide nanoparticles, such as aluminum, aluminum oxide, copper, silicon dioxide, titanium dioxide, and silver, as well as carbon-based nanomaterials, such as carbon nanotubes (CNT) and carbon black (CB) [2], Gold nanoparticle [7] , Silver nanoparticle [8].

Light is scattered by the interaction of the electrons with the incident radiation. The oscillating electric field causes a vibration on the electrons turning them into oscillating dipoles. These dipoles reemit radiation. As the electrons are moving sources (due to the Brownian motion) of radiation, the frequency of the radiation is shifted to higher or lower frequencies depending on its velocity and direction relative to the detector (Doppler Effect). Molecules in solution move in all directions with equal probability and have a continuous speed distribution, thus a continuous broadening of the spectrum, relative to the incident frequency line is observed. The power spectrum broadening is related to the Brownian motion of the particles in solution and hence to their diffusion coefficient, which in turn is related to the size and shape.

- ***Transmission Electron Microscopy***
The basic operation in a Transmission Electron Microscope (TEM) is that electrons generated from an electron gun are transmitted through the sample. The transmitted electrons are focused using electron optic lenses to form images. The imaging modes can be controlled by the use of an aperture. Since the attenuation of the electrons depends mostly on the density and thickness of the sample, the transmitted electrons form a two dimensional projection of the sample.

Sample preparation is an important step for the TEM analysis. The sample needs to be thinned to 40-150 nm thick to produce high quality TEM images. This can be achieved by techniques such as chemical polishing, electropolishing, mechanical polishing, dimpling, ultrasonic grinding, Ion thinning, fine particle dispersion and frozen hydrated film. The nanomaterials which are electron transparent such as powders or nanotubes can be quickly prepared by the deposition of a dilute sample containing the specimen onto support grids or films. The biological specimens can be prepared either by negative staining with uranil acetate or by plastic embedding to withstand the vacuum and facilitate handling. In materials science and metallurgy the specimens are naturally resistant to vacuum, but still must be prepared as a thin foil, or etched so that the specimen is thin enough for the electron beam to penetrate.

Figure 1 shows a series of images for a small Au particle recorded on videotape during an observation period of 20 s [11] Shape changes and different structural defects (see examples arrowed in frame f) are clearly evident. Figure 2 shows an example of a FeCo-filled nanotube produced at 700^0C [12]. TEM studies of GaN QDs, as illustrated by the truncated pyramidal dot visible in Figure 3. [13]
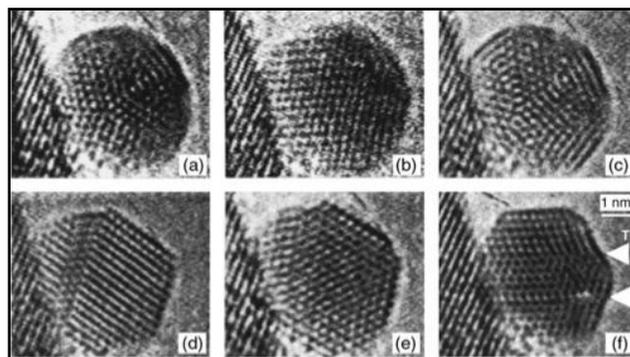
Fig. 1. Shape changes of a nanoscale multiply twinned particle of Au observed during a 20 s time interval during observation at very high magnification in a 400 kV HREM.
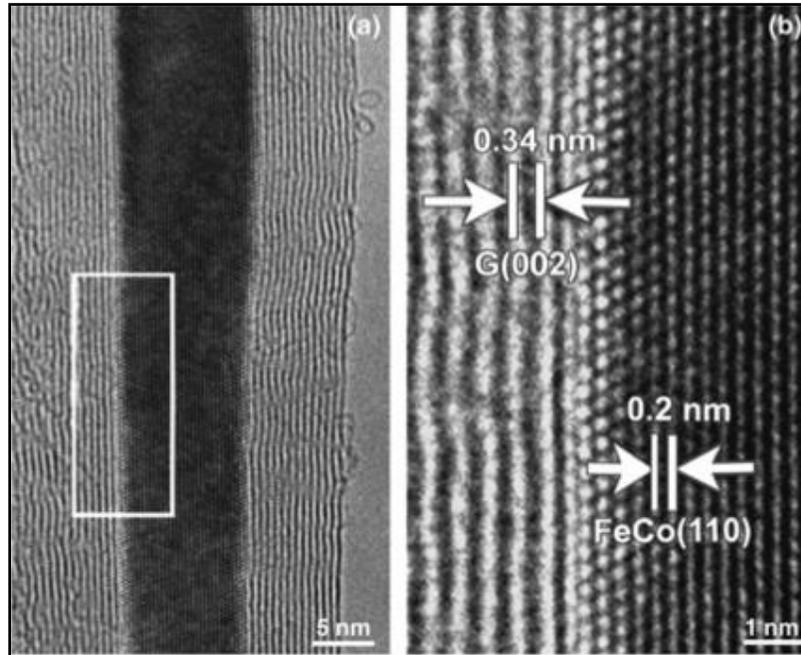
Fig. 2. (a) High-resolution electron micrograph of FeCo-filled multiwalled carbon nanotube produced at 700 ^{0}C; (b) Magnified view of FeCo alloy inside the carbon nanotube.
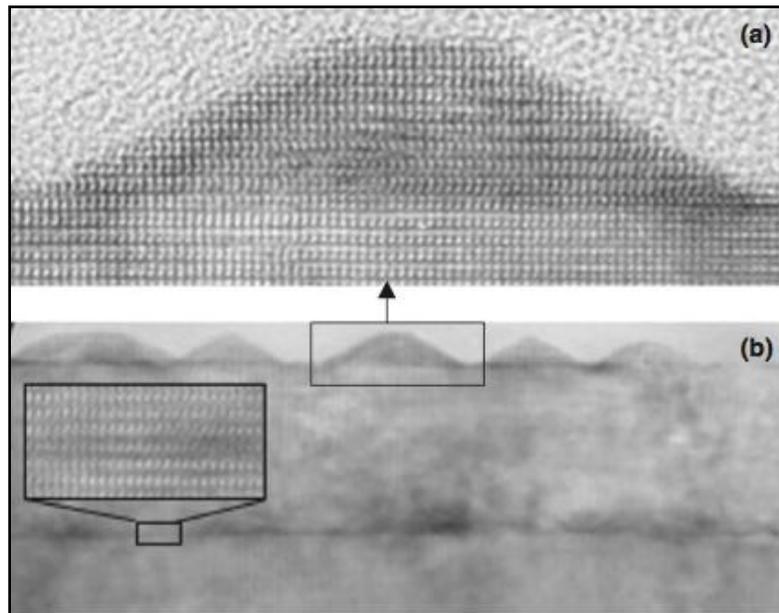
Fig. 3. (a) High-resolution electron micrograph showing cross section of GaN quantum dot with truncated pyramidal shape; (b) lower magnification view showing GaN wetting layer (inset) and GaN QDs grown on AlN barriers.

In spite of all these advantages, TEM imaging still presents a series of challenges. For instance, image overlap is a typical problem during observation. When this occurs, the surrounding matrix usually tends to mask the supported nanoparticles. In some special cases, however, the existence of an epitaxial relationship between the nanoparticles and their support can be used to

obtain size and shape information [14]. Moreover, nanoparticles can be susceptible to damage under the electron beam irradiation conditions normally used for high-resolution imaging.

An important precaution to be taken into consideration, when performing TEM measurements on nanoparticle-containing samples is that they can be susceptible to the highly energetic electron beam of the TEM instrument [15]. Beam susceptibility makes it very difficult sometimes to carry out electron diffraction studies on nanoparticles that are prone to beam damage. In this case, by using low electron beam currents, it is possible to obtain lattice fringe images and electron diffraction.

- *Scanning Electron Microscopy*

When the primary electron targeted over the sample surface, generate the secondary or backscattered electrons. These electrons are collected by a secondary detector or a backscatter detector, converted to a voltage, and amplified. The amplified voltage is applied to the grid of the CRT and causes the intensity of the spot of light to change. The image consists of thousands of spots of varying intensity on the screen of a CRT that correspond to the topography of the sample surface.

For conventional imaging in the SEM, specimens must be electrically conductive, at least at the surface, and electrically grounded to prevent the accumulation of electrostatic charge at the surface. Metal objects require little special preparation for SEM except for cleaning and mounting on a specimen stub. Nonconductive

specimens tend to charge when scanned by the electron beam, and especially in secondary electron imaging mode, this causes scanning faults and other image artifacts. They are therefore usually coated with an ultrathin coating of electrically-conducting material, commonly gold, deposited on the sample either by low vacuum sputter coating or by high vacuum evaporation. Conductive materials in current use for specimen coating include gold, gold/palladium alloy, platinum, osmium, iridium, tungsten, chromium and graphite [16]. Coating prevents the accumulation of static electric charge on the specimen during electron irradiation. An alternative to coating for some biological samples is to increase the bulk conductivity of the material by impregnation with osmium using variants of the OTO staining method (O-osmium, T-thiocarbohydrazide, O-osmium) [17, 18].

FE-SEM observations elucidate a variety of shapes in the 3D nanoparticle superlattices having well-defined or multiple facets, where many of them have plate-like or complicated morphologies Figure 4a. Magnified images Figure 4b of the plate-like superlattice marked with 1 clarified the hexagonal close-packed arrangement of gold nanoparticles with the core size of about 6 nm. In addition, the lattice image observed at the side facets confirmed the 3D ordered arrangement of the nanoparticles. Another example of a superlattice and its magnified surface images are shown in Figure 5a-c. In Figure 5c, a stacking fault (marked with "SF") as well as an excellent hexagonal particle arrangement could be observed. [19]
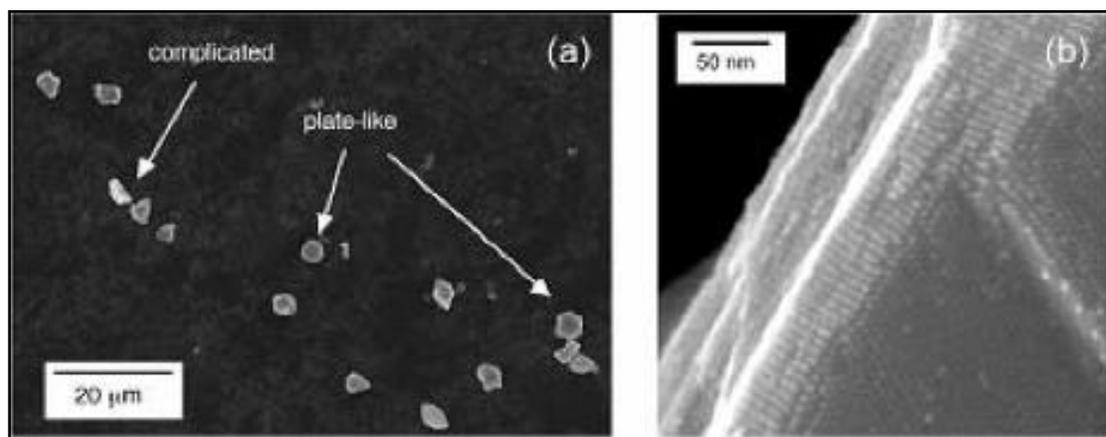
Fig. 4. (a) FE-SEM image of the superlattices of NAG-protected gold nanoparticles on a Si substrate. The superlattices show a variety of morphology having well-defined facets. (b) Magnified image of the superlattice marked with 1. [19]
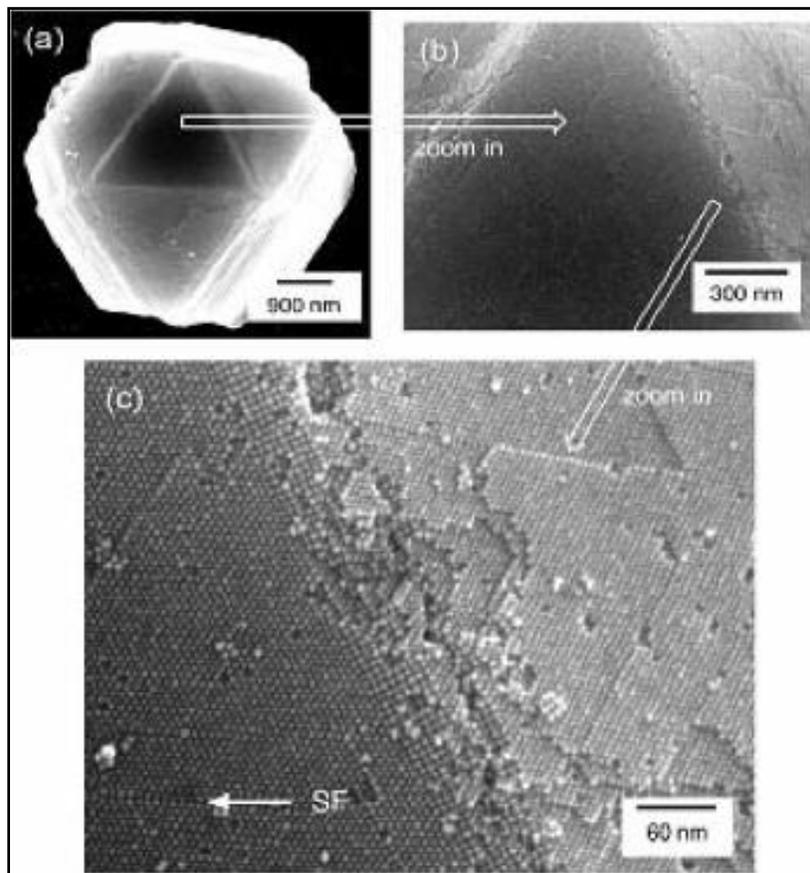
Fig. 5. FE-SEM images of a superlattice of NAG-protected gold nanoparticles. (b) and (c) show the magnified surface images of the superlattice. The image in (c) clarifies the hexagonal close- packed arrangement of nanoparticles and a stacking fault. An example of the stacking fault is marked with "SF". [19]

SEM is, to a certain extent, a limited tool to characterize nanoparticles. The main problem with the application of SEM to nanoparticle characterization analysis is that sometimes it is not possible to clearly differentiate the nanoparticles from the substrate. Problems become even more exacerbated when the nanoparticles under study have tendency to adhere strongly to each other, forming agglomerates. In contrast to TEM, SEM cannot resolve the internal structure of these domains but SEM and TEM in combination with Energy dispersive X-ray spectroscopy (EDS) can be used to carry out the elemental analysis of nanomaterials Figure 6.

The big disadvantage of both SEM and TEM in this context is that one can never be sure that the observed image is truly representative of the bulk nanoparticle sample. Consequently, bulk-sensitive methods that provide information regarding the quality, size, and structural properties of a given sample must be employed. Among these methods, Raman spectroscopy and optical absorption deliver the most comprehensive results.
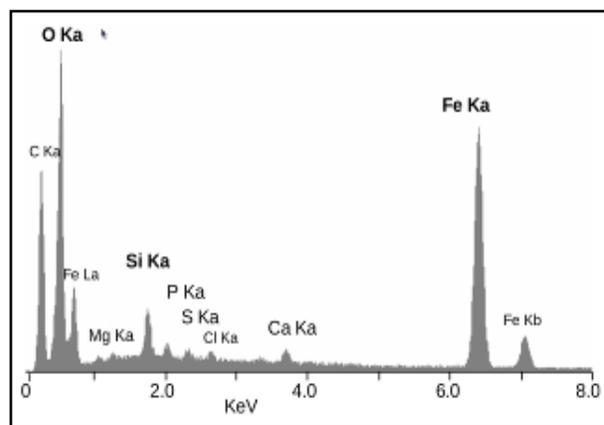
Fig. 6. A typical EDS profile of an Iron oxide sample [41]

- ### *Raman spectroscopy*

Among the several techniques used to characterize nanomaterials, Raman spectroscopy is perhaps the most powerful tool to get information on their vibrational and electronic structures [20]. Raman spectroscopy is based on the inelastic scattering of visible light by matter. Light scattering may be elastic or inelastic. Elastic scattering is the most common phenomenon and occurs without loss of photon energy (i.e., without any change in the frequency of the original wave). In contrast, a very small fraction of the incoming radiation undergoes inelastic scattering, in which the scattered wave compared with the incoming wave results in a different frequency. This frequency difference is called the Raman shift, which can be positive or negative. If, upon collision, the photon loses some of its energy, the resulting radiation has a positive Raman shift (Stokes radiation). In contrast, when the incoming photons gain energy, the resulting radiation has higher frequencies (anti-Stokes radiation) and a negative Raman shift is observed.

Another mechanism for the intensity enhancement of Raman signals is through the excitation of localized surface plasmons (SPs) [21]. Similarly in this case, it is necessary that the incident laser light used to obtain the spectra and the Raman signal is in or near resonance with the plasmon frequency [22]. This situation is satisfied normally for the case of organic molecules adsorbed on metals. The technique is then generally known as surface enhanced Raman spectroscopy (SERS).

Figures 7 and 8 shows an example of this approach – the results obtained by Njoki and colleagues for a set of SERS spectra for 4-mercaptobenzoic acid adsorbed on gold nanoparticles of different sizes in aqueous solution are presented [23]. The authors indicated that the size correlation with the intensity of SERS revealed that this intensity increases with particle size. Although there are many reports in the literature on SERS spectra for metallic nanoparticles, these focus on particles deposited on solid substrates [24, 25]. In this case, the observation of the SERS signals in the solution is noteworthy. The authors also analyzed the SERS intensity for the 1078 and 1594 cm−1 bands observed in Figure 9. Figure 10 shows this analysis; here the authors plotted the intensity against the particle size. These results are compared with the ones obtained for the values of

the absorption maximum on the optical absorption spectra of the gold nanoparticles, again against particle size. The authors indicated that in both cases, it is possible to correlate particle size either with the absorption maximum in the optical absorption spectra or with the SERS intensity. This size correlation of the SERS demonstrates the validity of determining the wavelength of the SP resonance band probed by SERS as a measure of the particle size.
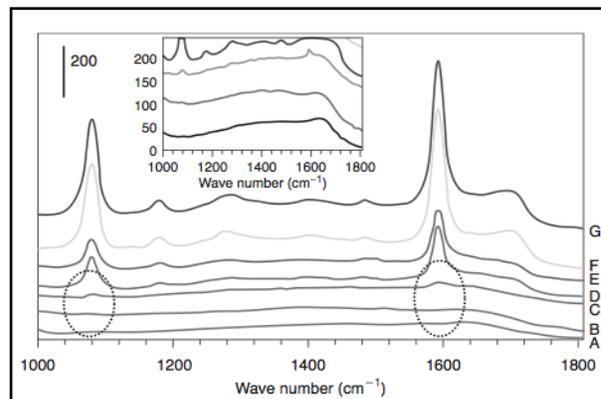
Fig. 7. Surface enhanced Raman spectra of 4-mercaptobenzoic acid adsorbed on gold nanoparticles of different sizes in aqueous solution (A) 30 nm, (B) 40 nm, (C) 50 nm, (D) 60 nm, (E) 70 nm, (F) 80 nm, and (G) 90 nm. (Inset): Magnified view of circled curves. Source: From Ref. 23.
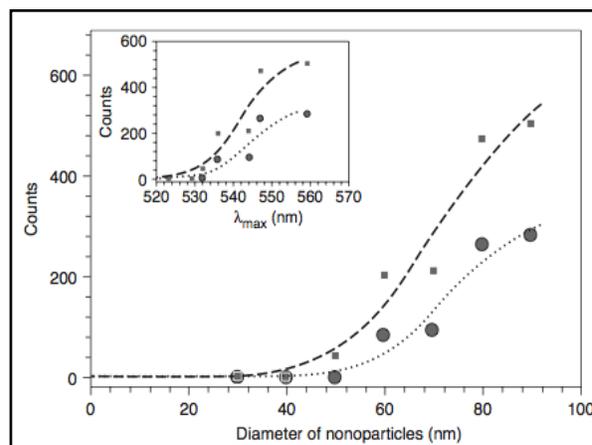
Fig. 8. Plots of the intensity of the surface enhanced Raman spectra. Plots of the intensity of the surface enhanced Raman spectra for 4-mercaptobenzoic acid adsorbed on gold nanoparticles from 1078 cm−1 (filled circles) and 1594 cm−1 bands (filled squares) as observed in Figure 6 against the particle size. The inset shows the results obtained by the authors when a similar correlation is performed using the maximum on the gold nanoparticles absorption spectra instead of the surface enhanced Raman intensity. Source: From Ref. 23.

A limitation of Raman spectroscopy is the extremely low quantum efficiencies associated with the process (almost 1 photon is inelastically scattered for every 106 photons that interact with the sample). Thus, a very intense light source must be used to get a signal strong enough to measure satisfactorily the Raman shift. Moreover, since a very precise measure of the frequency of the incoming light is needed to calculate the Raman shift, the use of a monochromatic excitation light is preferred. A laser light source satisfies both conditions: monochromaticity and high intensity; it is thus the obvious choice for excitation light source to perform Raman spectroscopy.

- *Optical spectroscopy*

A very effective analysis method that can be used to probe the size of nanoparticles is through their optical absorption spectra [26-28]. This technique is based on the well-known phenomenon of light absorption by a sample. In particular, the information obtained on the band energy gap is extremely useful to evaluate the dispersion and local structure of nanoparticles formed by d0 transition metal oxides, sulfides, and selenides [29-33]. Two recent studies report the development of experimental correlations between the size of gold nanoparticles and the concentration with its optical absorption spectra [23, 34]. However, these two methods seemed limited to particles with ideal spherical shapes. A more recent report provides quantitative relationships between Au nanoparticle size and concentration, accounting for the deviation of the particle size from ideal spheres [35].

This remarkable result (Figure 9) clearly showed that not only nanoparticle size but also nanoparticle concentration in liquid phases can be accurately determined from optical absorption spectra, provided that shape effects are taken into consideration [36, 37].

- *Atomic Force Microscopy*

Atomic force microscope (AFM) is now routinely used techniques for metrology and surface characterization of nanoparticles. Unlike other microscopy techniques, the AFM offers visualization in three dimensions.

The atomic force microscope (AFM) is a very high-resolution type of scanning probe microscopy which measures the force between the sample and the tip. Since no electric current is involved, the sample does not have to be metallic. There are two modes of operation: (1) Contact Mode: During contact with the sample, the probe predominately experiences *repulsive Van der Waals forces* (2) Non-contact mode: During contact with the sample, the surface *attractive Van der Waals forces* are dominant. It can achieve a resolution of ~1nm. The AFM can scan both hard and soft samples in ambient air or in a fluid environment. It is one of the foremost tools for imaging, measuring, and manipulating matter at the nanoscale. Figure 10 shows an AFM image of Al2O3 nanoparticles on a clean Si substrate.
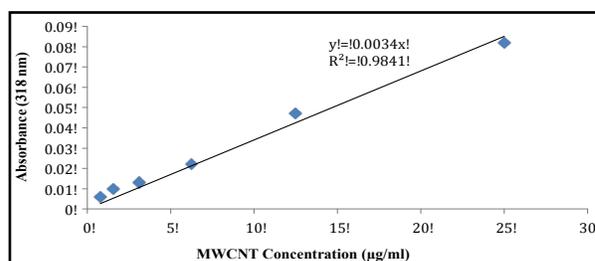
Fig. 9. Absorbance standard curve established from a suspension of MWCNT dispersed in BSA 1%, reproduced from Ref 37.
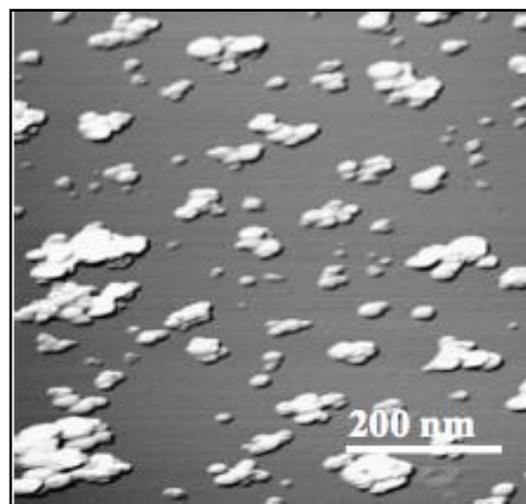
Fig.10. Al2O3 (0.02%) nanoparticles deposited on Silicon. Height of the nanoparticles is about 50nm. Reproduced from Ref [38].

There are three general rules to follow when using an AFM to image particles:
1. The particles must be rigidly adhered to a flat substrate.

2. The particles must be uniformly dispersed on the substrate.

3. The substrate roughness must be less than the size of the nanoparticles.

However there are limitations in achieving atomic resolution [39]. The physical probe used in AFM imaging is not ideally sharp. As a consequence, an AFM image does not reflect the true sample topography, but rather represents the interaction of the probe with the sample surface. This is called tip convolution. There are many different ways to analyze and characterize particles but there is not one single "best technique" for all situations [40]. Determining the best technique for a particular situation requires knowledge of the particles being analyzed, the ultimate application of the particles, and the limitations of techniques being considered Figure 11.
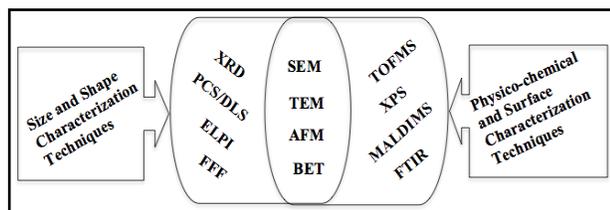
Fig. 11. Characterization and Detection Techniques of Nanoparticles.

Particle analysis techniques can generally be classified as ensemble and single-particle techniques. Ensemble techniques measure the response from statistically significant numbers of particles simultaneously. For Example, Dynamic Light Scattering, Laser Light Diffraction, Sedimentation techniques. Single-particle techniques isolate and identify data from individual particles. Statistical information from groups of particles can be obtained by processing the combined measurements of many different individual particles. Examples Light Microscopy, SEM, TEM, AFM.

Depending on the application of interest, a number of techniques can be used to analyze and characterize nanoparticles. In general, morphological information, such as shape and aspect ratio, as well as surface information, such as texture and roughness parameters, cannot be obtained using ensemble techniques. Only single-particle techniques, which look at individual

particles, can supply such information as summarized in Table 1.

Table 1. Techniques to analyze and characterize nanoparticles

Ensemble Analytical Techniques	Single-particle Techniques
Dynamic Light Scattering (DLS)/ (Photon Correlation Spectroscopy, PCS)	Electron Energy Loss Spectorscopy (EELS)
Electrical Low Pressure Impactor (ELPI)	Transmission Electron Microscopy(TEM)
Differential Mobility Analyser (DMA)	Atomic Force Microscopy (AFM)
Field Flow Fractionation (FFF)	Scanning Electron Microscopy (SEM)
Time of Flight Mass Spectroscopy (TOF MS)	Specific Surface Area (BET)
Fourier Transform Infrared Spectroscopy (FTIR)	
Centrifugal sedimentation	
X-Ray Diffraction (XRD)	

CONCLUSIONS

We conclude with the remark that the properties that must be measured for test materials used in nanotoxicity studies include size and shape, physical and chemical properties, surface area, and surface chemistry by using the techniques like TEM, SEM, AFM and BET. At the same time if the nanoparticle's surface is functionalized for dispersion then the state of dispersion, aggregation status also has to be studied by using the techniques like FTIR, DLS, SERS and EDS. The minimum characterization which is needed is summarized in the Figure 11 that has to be performed before any nanoparticle toxicological testing.

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