

# Methods to Characterize Nanoparticles for Mucosal Drug Delivery



**Kalpesh Vaghasiya, Ankur Sharma, Eupa Ray, Suneera Adlakha, and Rahul Kumar Verma**

**Abstract** Physicochemical properties of the nanoparticles are associated to their *in-vivo* behavior including pharmacokinetic, bio-distribution, efficacy, and toxicity profiles. It is imperative to gain a comprehensive understanding of the nanoparticle properties through their characterization. Characterization of nanomaterials depends upon their unique physical and chemical properties with different level of complexity at molecular levels. Distinct properties of nanoparticles often hinder when standard methods of characterization of particles are used, which compromise the reliability and reproducibility of the outcome. Nano-therapeutics characterization depends on various aspects, including the encapsulated drug, delivery vehicles, disease, route of administration, dosing amount and its application. The precise control over nanoparticle properties need robust and advanced characterization techniques. Generally, characterization of nanoparticles is based on the composition, size- distribution, morphology, surface charge, purity and stability, using sophisticated techniques such as dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM) etc. Mean particle size, morphology and surface charge of nanoparticles affect their physical stability, re-dispersibility and *in-vivo* biodistribution. This chapter summarizes the basic principles, associated challenges and practical concerns in standard and promising physicochemical techniques used for characterization of nanoparticles.

**Keywords** Nano particles · Mucosal delivery · Characterization · Composition · Morphology · Size · Charge · Interaction · Stability

---

K. Vaghasiya · A. Sharma · E. Ray · S. Adlakha · R. K. Verma (✉)  
Institute of Nano Science and Technology (INST), Mohali, Punjab, India  
e-mail: [rahulverma@inst.ac.in](mailto:rahulverma@inst.ac.in)

## 1 Introduction

Nanotechnology has several conceivable benefits to pharmaceutical research by making medicines more effective and minimizing their side effects. Nanoparticles have wide ranging implications in diverse field of science and technology including medicine, biotechnology, material science, etc. [1]. Designing precise drug delivery systems has long been a major challenge for pharmaceutical researchers. Further, drug delivery via mucosal sites offers several advantages over the traditional parenteral administration. However, it is challenging for several nanoparticulate formulations to cross the mucosal barrier and reach their target site in the deeper tissues or after subsequent absorption to the systemic circulation. Thus, evolving nano-formulation technologies explore several strategies that would enhance interaction with the mucus surface and the epithelial cell layer in order to achieve high drug levels at the target site [2]. Therefore, comprehensive knowledge about nanoparticle characterization is essential in order to improve current approaches and to develop new delivery systems to lower the barrier for improving mucosal delivery. In order to overcome these barriers, nanoformulations need to have several features like mucoadhesive properties, membrane permeation, cellular uptake augmenting, and drug release governing properties.

In order to gain a comprehensive understanding of the composition and nature of the nanoparticles, researchers first need to have enough information on the available nanoparticles characterization techniques. Characterization of nanoparticle proposed for therapeutic or diagnostic applications is complicated due to the wide variability of materials used for their preparation. Further, the multi-functionality and distinctive surface properties of nanoparticles makes characterization even more difficult by standardized methods. Several nanoparticles like colloidal gold-particles, silver-particles, quantum-dots etc. have optical properties which interferes with calorimetric assays and potentially give false positive results [3]. Some nanoparticles like dendrimers, silicon, cadmium selenide, titanium dioxide etc. can have catalytic properties that interfere with enzymatic testing of nanoparticles. Polymeric nano-formulations may contain variable concentrations of surfactants to improve stability and dispersibility in liquid. The surfactant alters the surface tension of the medium and hence interferes in their characterization. Nanoparticles readily absorbs impurities from the medium due to their large surface area and surface charge, which gives inconsistent analytical results. Selectively delivering drugs to targeted disease site using nanoparticles can help to enhance the therapeutic effectiveness and reducing adverse effects in normal tissues. Several advanced nanoparticles are functionalized by specific targeting moieties that facilitate their precise recognition and effective delivery to target cells. These targeting moieties includes proteins, antibodies, peptides (arginine-glycine-aspartate; RGD), aptamers, polysaccharides, glycoproteins, folate etc. which are broadly used in developing multifunctional nanoparticles. However, the complexation of nanoparticles with targeting moieties further increase the complexity of delivery system and make their

**Table 1** Different methods to characterize nanoparticles

Parameters	Techniques
Particle size distribution	Dynamic light scattering (DLS), photon correlation spectroscopy (PCS), scanning electron microscopy, transmission electron microscopy, atomic force microscopy
Surface charge (zeta potential)	Laser doppler anemometry, Zeta potentiometer
Shape	Scanning electron microscopy, transmission electron microscopy, Atomic force microscopy, Coulter counter
Surface hydrophobicity	X-ray photoelectron spectroscopy, water contact angle measurement Hydrophobic interaction chromatography
Surface properties	Static secondary-ion mass spectrometry
Surface area	Brunauer–Emmett–teller (BET) Analyzer
Crystallinity	X-ray diffraction, Small angle X-ray scattering (SAXS), differential scanning calorimetry thermal analysis (DSC-TGA)
Density	Helium compression pycnometry
Drug loading and release	Dialysis membrane <i>in-vitro</i> drug release assay using analytical techniques, such as HPLC, UV, <i>etc</i>

characterization more challenging. These complications therefore tend to impede the development of standard characterization methods for nanoparticles.

For nanomaterials, unfortunately there is a lack of known regulatory protocol and standardized set of detection and characterization methods [4]. Nanoparticle researchers usually establish their own characterization and quality control methods for their nanoparticles. Regulatory authorities therefore face various issues in interpreting and evaluation of characterization data without any substantial reference to published literature. These issues further complicate the approval process for nanoformulation for diagnostics and therapeutics.

Physicochemical characterization of nanoparticles such as shape, size, surface area, chemical composition, aggregation, surface functionality, stability etc. can provide better understanding of structure–activity relationships and biological activity *in vivo*. In this chapter, we will provide a comprehensive description of various nanoparticle characterization technique and are out lined in Table 1 [2, 5, 6].

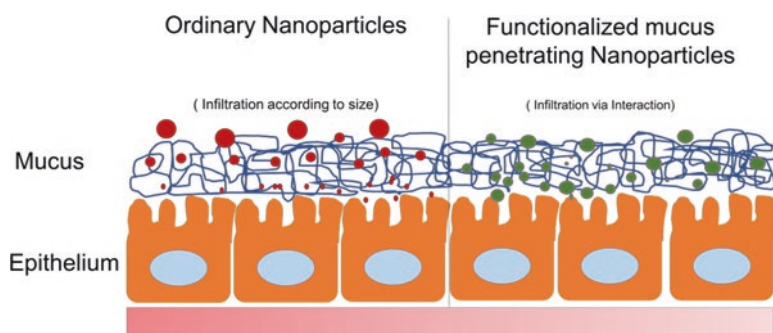
## 2 Nanoparticle Characterization Parameters

The basic of nanotechnology lies in the fact that properties of materials change significantly when particle size is reduced to nanoscale range. The behavior of nanoparticles is fundamentally different from their bulk counterparts due to the change in surface-to-volume ratio and enhancement of quantum properties. However, measuring this aspect is difficult and pose challenges to researchers. Physicochemical properties, like particle size, surface zeta potential, shape, surface

properties, adsorption potential, molecular weight, composition, solubility, stability, purity, identity, aggregation, wettability, porosity, distribution of conjugated moieties, and impurities are critically relevant to physiological interaction in biological systems. It is therefore crucial to understand various nanoparticle properties based on their different features by reliable and robust characterization techniques: as *in-vivo* physiological interaction behavior of particles may influence the therapeutic efficacy and diagnostic accuracy in medical applications.

## 2.1 Particle Size Distribution

Most of the unique properties of nanoparticles are size-dependent which do not exist unless the size of the particle is decreased to nano-dimensions. The surface-to-volume ratio along with quantum effects of nanomaterials exhibits several size-dependent phenomena like optical, electronic, magnetic and mechanical properties. The particle size plays an important role in inherent nanoparticle properties and hence it is an essential task in the characterization of nanoparticles. The particle size distribution (PSD) of the nanoparticle governs biological fate that could affect physiological processes including deposition, distribution, targeting, metabolism and toxicity. For the development of drug delivery systems, the size of nanoparticles plays a major role as particle has to navigate from the administration site to the targeted site via various biological barriers. Subsequently, after administration of the nanoparticle formulation, it undergoes a biodistribution step and reaches the different organs or target site according to their size. To achieve better biodistribution of drug encapsulated in nanoparticles via crossing epithelial barriers to the target site, nanoparticles  $<1\ \mu\text{m}$  are preferred. Depending upon their size, nanoparticles could escape from the systemic circulation through openings of endothelial barrier called as fenestrations. It has also been demonstrated that nanoparticles 200 nm or larger could activate the lymphatic system and are cleared from circulation faster. Micron sized particles (1–5  $\mu\text{m}$ ) are majorly cleared through mononuclear phagocytic system cells, whereas 150–300 nm sized particles could be found in liver and spleen. Nanoparticles of 30–150 nm may also accumulate in the heart, kidney, and bone marrow. Smaller nanoparticles (5–10 nm) are rapidly cleared from systemic circulation while 10–70 nm diameter nanoparticles may penetrate capillary walls throughout the body [7]. In order to cross the endothelial barrier, nanoparticles should have size smaller than 150 nm. Desai et al. 1997 reported that nanoparticles of size 100 nm demonstrated a 2.5-fold more cellular uptake as compared to a 1  $\mu\text{m}$  particle, and 6-times greater uptake than a 10  $\mu\text{m}$  particles [8]. In various pathological conditions, vasculature structure undergoes several modifications, as in tumor grows where neo-vascularisation occurs and endothelium structure becomes discontinuous resulting in passage of larger nanoparticles (200–780 nm) across the barrier [9, 10]. Therefore the nanoparticles need to have an optimum size which can deliver sufficient drug and further evade the immediate clearance by the lymphatic system.



**Fig. 1** Translocation permeability of nanoparticulate drug delivery systems across mucosal barriers

For mucosal delivery, particulate delivery systems could be trapped in mucus layers (lung airways, gastrointestinal tract, vagina, eye etc) by steric barrier or adhesion. The thickness of the mucus layer in humans could vary depending on its site: for instance stomach has 50–600  $\mu\text{m}$  and 15–450  $\mu\text{m}$  in intestine [11]. These particles are normally removed quickly from the mucosal tissue and hence prevent delivery of drugs in these areas. Mucus is a dense porous structure composed of cross linked mucin fibers by hydrophobic interactions. Mucus show diverse pore size ranging from  $\sim 10$  to 1800 nm depending on its site in the body, for example, the mean mesh pore size of human intestinal mucus pore size  $\sim 200$  nm, human vaginal mucus  $\sim 350$  nm, cystic fibrosis lung mucus  $\sim 140$  nm, bovine vitreous  $\sim 500$  nm. Hence, in order to penetrate and infiltrate mucus, particles need to have an optimum size to evade any obstruction [5, 12] (Fig. 1).

## 2.2 Surface Area

Surface area is another important parameter for therapeutic nanoparticles as it affects reactivity and surface interactions with ligands. A decrease in particle-size leads to an exponential increase in surface area, and an increase in the availability of reactive surface. The high surface area of drug delivery systems can be achieved either by making small particles where the surface-to-volume ratio of particle is high or by developing materials with large number of voids compared to bulk material. Nanoparticles having high surface-to-volume ratio results in augmented surface reactivity, enhanced rate of dissolution, improved bioavailability and altered pharmacokinetic and toxicity profile. The interactions of nanoparticles with cells or microorganisms generally take place at the particle's surface, so the surface area is a major factor for possible therapeutic effects of nanoparticles. The larger surface area of nanoparticles dramatically enhances the equilibrium solubility, the rate of dissolution and generation of reactive oxygen species [13]. Further, the large surface

area allows for outer surface functionalization and can be done specifically for a particular receptor.

### 2.3 *Shape*

The shape of the nanoparticles is also an important design parameter that affects various biological phenomenon like movement of particles in systemic circulation, internalization by cells, physiological efficacy and degradation. Furthermore, the *in-vivo* circulation of nanomedicine can be altered by modifying the shape of drug loaded nanoparticles. Most of the nanoformulations are prepared in spherical shapes but advanced fabrication approaches have permitted the manufacture of wide-ranging shapes of nanocarriers with high precision. These shapes include rods, cubes, disks, ellipsoids, cylinders, hemispheres, cones, chains, biconcave discoids, dendrites etc. Irregular shapes show noteworthy influence on their transport through systemic circulation, their half-lives, cellular uptake and following intracellular targeting. Recent literature demonstrates the significance of shape of nanocarrier on many biological processes. Though, spherical structure is the most common shape in use, asymmetrical shapes could also be favorable in several occasion to enhance circulation time of the particles in vessels with decreased collisions against the vessel walls [14]. Asymmetric nanoparticles show different hydrodynamic behavior compared to spherical particles and are less susceptible to phagocytic clearance by the macrophages, which ease better sustained delivery of drugs [14].

Non-spherical systems demonstrated varied biodistribution profiles compared to their spherical counterpart, providing a different approach for targeting specific sites. Yu et al. 2016 demonstrated that the nanoparticle shape can have significant role in the mucus-penetrating abilities. They showed nanorods penetrates faster in GIT mucus of rat as compared to spherical nanoparticles of equivalent size. This phenomenon was attributed to rotational movement enabled by the flow and the mesh size of mucus [15].

### 2.4 *Zeta Potential*

Dispersed nanoparticles in solution are located at different locations in the diffuse layer of liquid due to the electric potential difference, so the movement of particles in this layer of liquid is called slipping and shear plane. The measurement of potential at this plane is called zeta potential [16]. The zeta potential of the nanoparticles is an important factor as it affects the particle dispersion characteristics and influences the adsorption of ions and biomolecules. The surface charge of nanoparticles is approximated by zeta potential measurements which is the function of surface charge of nanoparticle, adsorbed molecules on its surface, and the ionic strength and composition of the surrounding solutions. Therefore, the storage stability of

colloidal nanoparticles dispersion is mainly dependent on its zeta potential. Electrostatic interaction of nanocarriers is controlled by modification in their surface charges, that is analyzed by measuring the zeta potential of nanoparticles [17]. To achieve higher stability and to avoid aggregation of nanoparticles, higher value of zeta potential (either negative or positive) is preferred. The measured value of zeta potential indicates the surface hydrophobicity, the nature of the material encapsulated in nanoparticles and coating properties of the surface [13, 18]. Surface of nanocarrier is an important concern in targeting drug delivery systems. Un-functionalized nanoparticles having neutral or negative charge are swiftly opsonized and cleared by macrophages. Surface functionalization of nano-formulations is a common practice to evade the opsonization phenomenon and to maintain sustained drug delivery in systemic circulation. Appropriate zeta potential can enhance the drug targeting, its release profiles and stability of the nano-particulate formulations. It is demonstrated that nanoparticle with high surface charge and large particle size are engulfed more efficiently by macrophage. Small difference in surface charge and size has significant effect on cellular uptake. Nanoparticles with adequate hydrophilicity and uncharged surface can efficiently diminish the positive interactions between mucus and particles by decreasing electrostatic interactions.

## ***2.5 Surface Properties***

The surface properties of nanoparticles are functions of molecular or atomic compositions, charge and functional groups present on the surface which are responsible for the interaction with the surrounding environment. The surface properties of nanoparticles are intrinsically relevant to the superficial layer, but not to the overall bulk material. Surface properties of nanoparticles have potential effects on physiological barrier penetration, receptor binding, dispersion stability and aggregation. The manipulation of surface properties of nanoparticles is another prospect to design superior nanocarrier systems. To design an ideal nanocarrier system, the functionalization of particle surface with suitable targeting moieties, modification of surface hydrophobicity and reactivity can be useful to address aggregation, stability, and receptor binding. In multifunctional nanoparticles, functional moieties are conjugated with the surface to bind to target receptor in specific tissues/organs. The selection of materials and their surface properties like hydrophobicity or cross-linking density can be important factors for the designing of a mucus penetrating particles. Pharmaceutical scientists have developed several surface engineering strategies to generate hydrophilic coating and lessen the particle adhesion with mucus. Despite having negative charge on their surface, hydrophobic nanoparticles like polystyrene particles diffuse into mucin hydrogel by hydrophobic interactions. Researchers have proposed various active targeting strategies to increase nanoparticle penetration through mucus, mucoadhesion and cellular uptake by covalently functionalizing specific targeting moieties on the surface of the nanocarriers [2, 12]. Lectins are commonly used ligands for enhanced mucoadhesion and cell



internalization via specific cell interactions [19]. Another promising strategy to increase nanoparticle penetration through mucus is the incorporation of mucolytic-enzymes on the surface of the particles [20]. The characteristic ability of multifunctional nanoparticles to combine therapeutic, targeting and imaging modalities is a key aspect of their versatility and anticipated specific clinical impact [15].

## **2.6 *Composition and Purity***

A vast variety of nanomaterials are being used to design and develop nanoformulations for therapeutic purpose. These nanomaterials includes polymers, lipids, proteins, DNA, metal and metal oxides, inorganic minerals or other organic compounds. The composition and purity of nanoparticles majorly influences the transport, delivery, pharmacokinetics and biodistribution. In therapeutic applications of nanoparticles, it is common to combine two or more types of materials to form a complex or conjugant. Each ingredient exhibits their own inherent physicochemical properties including solubility, size, shape, surface charge, hydrophobicity and aggregation tendencies which are designed for different therapeutic response. The potential interaction of these materials with biological systems like cell and tissues is relatively different from each other depending upon the nature of material. Hence efficacy and toxicity significantly depends on the actual composition of the nanoparticle formulation. The measurement of chemical composition of multifunctional nanocarriers is more complicated compared to single entities. The occurrence of impurities in nanoformulations may considerably affect efficacy or introduce adverse effects. In accordance, the purity analysis of nanoparticles through their chemical composition is necessary. Prior to nanoparticle formulation synthesis, proper purification processes must be performed to remove side products, residual manufacturing components and endotoxin contamination. The purity analysis of nanoparticles must be carried out to check the presence of solvents, chelates and free metals, precursors, dimers, unconjugated therapeutics or other agents. Appropriate methods and techniques to detect the presence of such unwanted entities are required to ensure the quality and purity of the nanoparticle preparations [4, 10].

## **2.7 *Stability***

Pharmaceutical stability refers to the retaining of the bio-physico-chemical properties for a stated period of time after its manufacturing. Various factors affecting the conventional single molecule pharmaceuticals stability are similar for the conventional nanoparticles, including moisture, temperature, particle/molecular size, pH, solvents, enzymatic degradation, exposure to different types of ionizing and non-ionizing radiation, and presence of other excipients and impurities [4]. The multifunctional nanoparticles have complex compositions, so the stability of all the



components included in nanoparticles is essential to achieve its potential biological function. If any of the components of the complex will prematurely release, then it will compromise the efficiency of the nanoparticles activity. Therefore, it is necessary to evaluate the *in vitro* stability of functional components in various physiological conditions including pH, temperature and ionic strength. Further, stability evaluation in non-physiological conditions is also important to check the effects of short term and long term storage, ultrafiltration, lyophilization, freeze-thawing, pH variation, thermal and light exposure [10].

## 2.8 Drug Release

Drug loading is the amount of drug bound or encapsulated per total mass of polymer. Drug loading capacity of nanoparticles and its release influences the dose of the drug. The drug release from the nano-formulation depends upon several aspects of the material including matrix, porosity, matrix degradation, pH, temperature etc. Further, drug release rate from nano-formulations depends on the solubility of drug, desorption of the adsorbed drug, diffusion via matrix, degradation and diffusion process. Determination of extent of drug release from nanoparticles and subsequent biodegradation of the matrix is very essential for development of successful nano-formulations. The amount of drug is quantified by the use of UV-Vis spectroscopy, high performance liquid chromatography (HPLC), or liquid chromatography mass spectrometry (LC-MS) [18]. The *in vitro* drug release from nanoparticles is determined by various methods like dialysis bag diffusion technique, side-by-side diffusion cells with biological or artificial membranes, reverse dialysis sac technique, ultrafiltration, ultracentrifugation, and centrifugal ultrafiltration. The release profile of drugs from nanoparticles depends upon the nature and type of the drug delivery system [21, 22]. When the drug is uniformly dissolved or distributed in the polymer matrix (e.g nano-spheres), the release majorly occurs by erosion or diffusion of the matrix. The adsorbed or weakly bound drugs on the large surface area of nanoparticles are rapidly released (burst released) compared to the incorporated drug. In heterogeneous systems (e.g nanocapsules), the release of drug is mainly governed by diffusion over the polymeric degradation mechanism. The rate of diffusion of drug is faster as compared to matrix degradation, and is less dependent on the type of polymer.

## 3 Methods of Characterization of Nanoparticles

The characterization of nanoparticles is mainly performed for the measurement of their size distribution, shape, morphology, average particle diameter, charge affects, surface and elemental analysis, thermal stability and optical properties. Size and surface morphology are determined by techniques such as transmission electron

microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM) and dynamic light scattering (DLS). Stability and shelf-life of nanomedicine depends upon surface properties, surface charge, composition and storage conditions. Other techniques like ultraviolet-visible spectroscopy (UV-Vis), X-ray diffraction (XRD), small angle X-ray scattering (SAXS), fourier transform infrared spectroscopy (FTIR), mass spectrometry (MS), differential scanning calorimetry (DSC), thermo-gravimetric analysis (TGA), etc. will also be discussed. On the basis of characterization techniques and instrumentation, nanoparticle analysis can be categorized as described in the below sections.

### 3.1 Particle Size and Morphology Analysis

#### 3.1.1 Dynamic Light Scattering (DLS) or Photon-Correlation Spectroscopy (PCS)

The therapeutic properties of nanoparticles are highly dependent on their size and their tendency to agglomerate. Various techniques like sieve analysis, electro resistance counting methods, optical counting methods, sedimentation techniques, acoustic spectroscopy, dynamic light scattering, laser diffraction methods, etc. are all useful for particle size determination. Amongst them dynamic light scattering (DLS) is most popular and frequently used technique for obtaining size distribution of nanoparticles [23]. DLS can measure particle size distribution of small particles or polymers at the submicron or nanometer scale in suspension, and emulsion form by using a monochromatic light source, *e. g.* laser. Nanoparticles in the liquid phase experience brownian motion which is inducted by the bombardment of solvent molecules. Monochromatic light exposure hits the moving nanoparticles in solution which leads to a shift in incident light wavelength (at a fixed scattering angle) and the extent of the shifting in wavelength is due to interferences of the scattered light that measures the size of the particle (Fig. 2A). When moving particles are illuminated with laser, the intensity of light fluctuates which depends upon the size of the particle. This random motion of the particle is modeled by the Stokes-Einstein equation which links diffusion-coefficient measured by dynamic light scattering to particle size. This formula is most often used for particle size analysis.

$$D_h = \frac{k_B T}{3\pi\eta D_t}$$

$D_h$ : Hydrodynamic diameter

$D_t$ : Diffusion coefficient.

$k_B$ : Boltzmann's constant

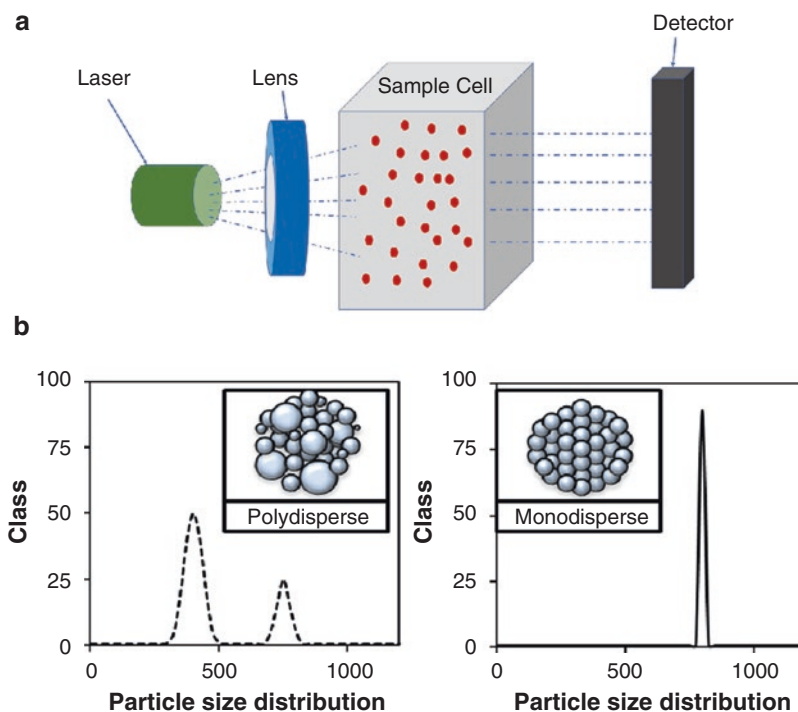
T: Temperature.

$\eta$ : Solvent viscosity.

The major advantage of DLS is its short experimental time duration, ability to characterize diluted samples, accuracy in measurement of the hydrodynamic diameter of the monodisperse/polydisperse sample (Fig. 2B), lower apparatus cost and reproducible results and analyzing samples in a wide range of concentrations. However, DLS has limited utility for investigation of heterogeneous size samples and resolving the dimension measurements of a mixed sample population. It is also not suitable for accurately measuring the sizes of non-spherical nanomaterials. Griffiths et al. evaluated the interaction between nanoparticles and mucin, where they demonstrated that negatively charged and hydrophilic nanoparticles did not display any interaction with mucin while positively charged and hydrophobic nanoparticles illustrated a strong interaction. This study showed that the DLS technique is a potential screening tool to nanoparticle-mucin interactions [24].

### 3.1.2 Scanning Electron Microscopy (SEM)

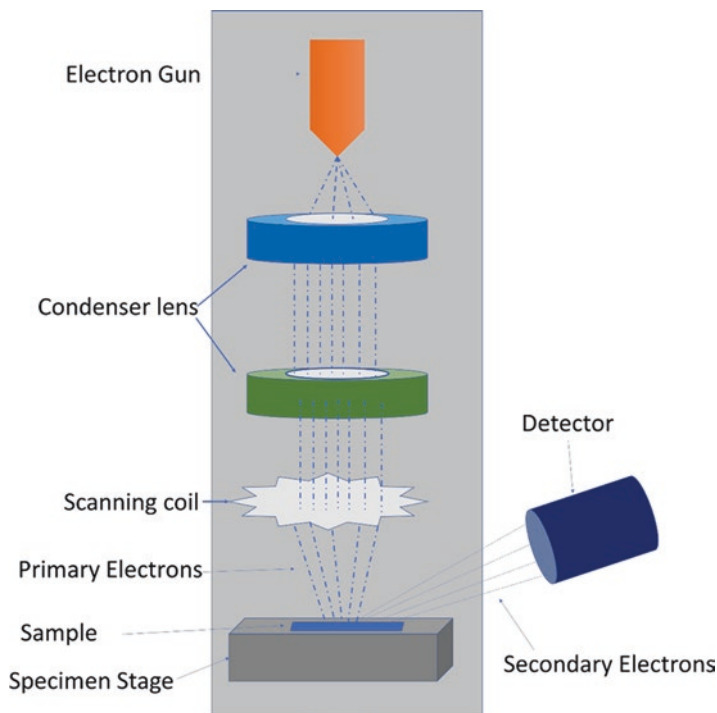
Scanning electron microscopy determines the size, shape, surface morphology of agglomerated/ dispersed nanoparticles, and surface functionalization with direct visualization of the nanoparticles [18]. It shows detailed three-dimensional images



**Fig. 2** (A) A schematic diagram of a dynamic light scattering instrument (B) Graph of two nanoparticle batches of a bimodal polydisperse population and a monodisperse population obtained after analysis by light scattering

of high magnification particles (upto 300,000X). The mean particle size obtained by SEM is comparable with the size obtained by dynamic light scattering. In contrast to optical microscopy, that uses a light source and lenses to observe the samples and to generate magnified images, SEM uses beams of accelerated electrons to generate higher magnification images. For SEM analysis, suspension of particles is mounted on a sample holder and dried, followed by coating of conductive metal (e.g. gold) using a sputter coater under vacuum. Nanoparticles are evaluated by scanning with a beam of electrons, and the secondary electrons/backscattered electron/characteristic X-rays generated from the specimen surface reflects the atomic composition and topographical information of the particles [25] (Fig. 3).

X-rays are the second most common imaging mode for SEM analysis, which gives information of element composition of nanoparticles. The specific technique is known as Energy Dispersive X-Rays (EDX) SEM. Back scattered electron generated from samples are also occasionally in SEM analysis for elements. The image that is displayed on the monitor is the distribution map of the intensity of the signals emitted from the scanned part of the sample. The major limitation of SEM is that it requires conductive surface of the sample to scan the surface by an electron beam. Many biological molecules and polymers have nonconductive surfaces that may acquire a static electric charge and insufficiently deflect the electron beam which



**Fig. 3** Schematic of Scanning electron Microscope (SEM)

tends to generate artefacts or imaging faults. Therefore, nonconductive samples are coated with an ultrathin layer of electrically conductive material under high vacuum evaporation or low vacuum sputter coating.

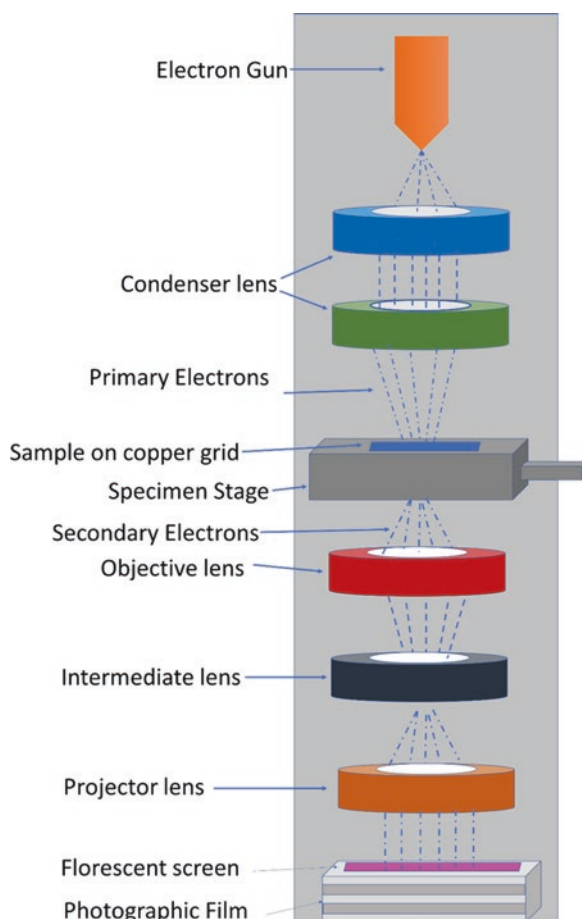
Nonconductive samples may also be imaged by specialized “Environmental SEM” (ESEM) or in field emission gun SEM which is operated at high vacuum, low voltage or at high voltage, low vacuum [4]. Sometimes, electron beam can damage the nanoparticles or the biological samples. The process of drying and contrasting of nanoparticles may also cause shrinkage of the sample and therefore change the characteristics of the nanoparticles. More sophisticated instruments such as, field emission SEM uses narrower probing beams at high and low electron energy which gives better spatial resolution while reducing the sample destruction [25, 26]. However, this method is time consuming, expensive and often needs additional information about size distribution.

### 3.1.3 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is a frequently used technique for the characterization of nanoparticles. It provides direct images and elemental information of nanoparticles at a spatial resolution down to the level of sub-nanometer/atomic dimensions. In the conventional TEM mode, an electron beam emitted from a source is accelerated at high voltage potential and transmitted through an ultrathin foil specimen. The incident electrons interact with the sample and transform to either unscattered electrons or elastically/inelastically scattered electrons [27]. The scattered or unscattered electrons are focused through various electromagnetic lenses and then projected on a screen to obtain electron diffraction pattern, which forms a phase contrast image, amplitude contrast image or a shadow image according to the density of unscattered electrons. The image is magnified and focused by adjusting the ratio of the distance between the specimen and objective lens (Fig. 4). Newer TEM are specifically equipped with the specimen holder which allows tilting the specimen at different angles in order to get specific diffraction patterns [28]. A range of analytical techniques can be coupled with TEM for different type of applications, for example (i) electronic structure of the nanoparticles that can be quantitatively investigated by chemical analyses of electron energy loss spectroscopy and (ii) chemical composition of the nanoparticles can be quantitatively investigated by energy dispersive X-ray spectroscopy [25, 28]. The TEM is widely applicable in biological sciences, material sciences and metallurgy. However, there are certain drawbacks of TEM, it requires thin layer samples (to transmit sufficient electrons to produce images) and high vacuum conditions which may lead to sample destruction. The preparation of extensive thin specimen enhances the chances of altering the structure and makes analysis a time consuming process.

The nanoparticles dispersion is deposited onto the support grids or films for characterization. TEM is useful in the measurement of particle size, aggregation/agglomeration, and dispersion, of nanoparticles (Fig. 5).

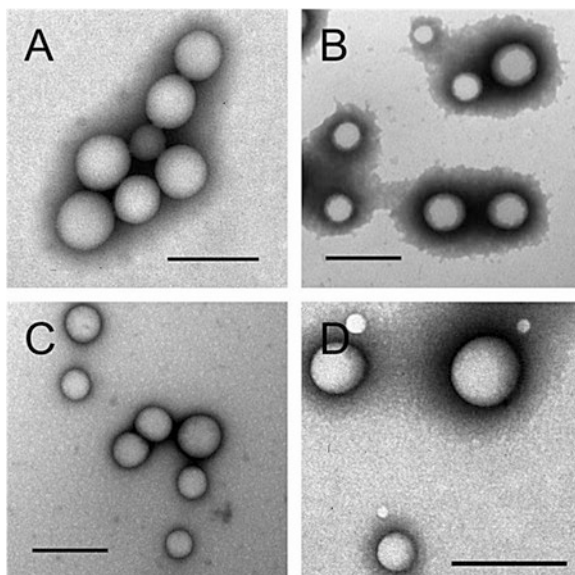
**Fig. 4** Schematic diagram of transmission Electron Microscopy (TEM)



### 3.1.4 Atomic Force Microscopy (AFM)

In contrast to electron microscopy technique, AFM is a scanning probe technique/ scanning force microscopy, which can divulge a range of information regarding the nanoparticles or biomolecules and its interaction on a single particle basis. The reported resolution of fractions of nanometer that is more than 100 times better than to optical diffraction limit. AFM is ideal for quantitative measurement of surface roughness and visualizing surface nano-texture of nanoparticles. It also helps in the determination of the size, shape, structure, aggregation and dispersion of nanoparticles. Due to its non-destructive analysis and high 3D spatial resolutions, it is a very useful tool in the analysis of conductive/nonconductive, dry/wet, soft/hard, or any other type of material in physiological conditions [25, 29]. AFM consist of a silicon/silicon nitride micro machined cantilever with a sharp tip (radius  $\sim 10$  nm) attached at one end to detect whether the deflection of the cantilever tip occurred due to van-

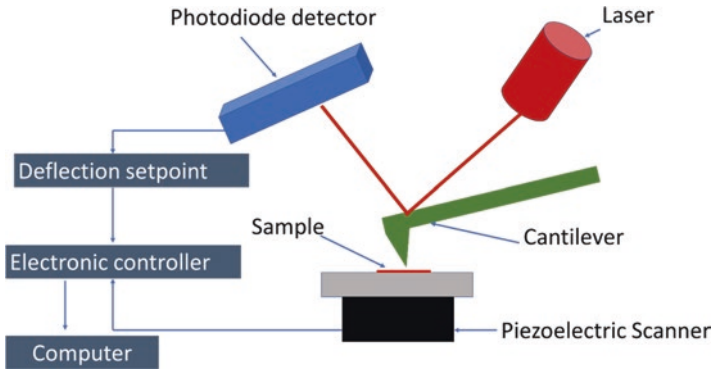
**Fig. 5** PLGA nanoparticles observed by TEM: (A) stabilizer-free PLGA, (B) PLGA/PVA, (C) PLGA/Chitosan and (D) PLGA/PF68 (bar = 500 nm) (Reprinted with permission from ref. [26])



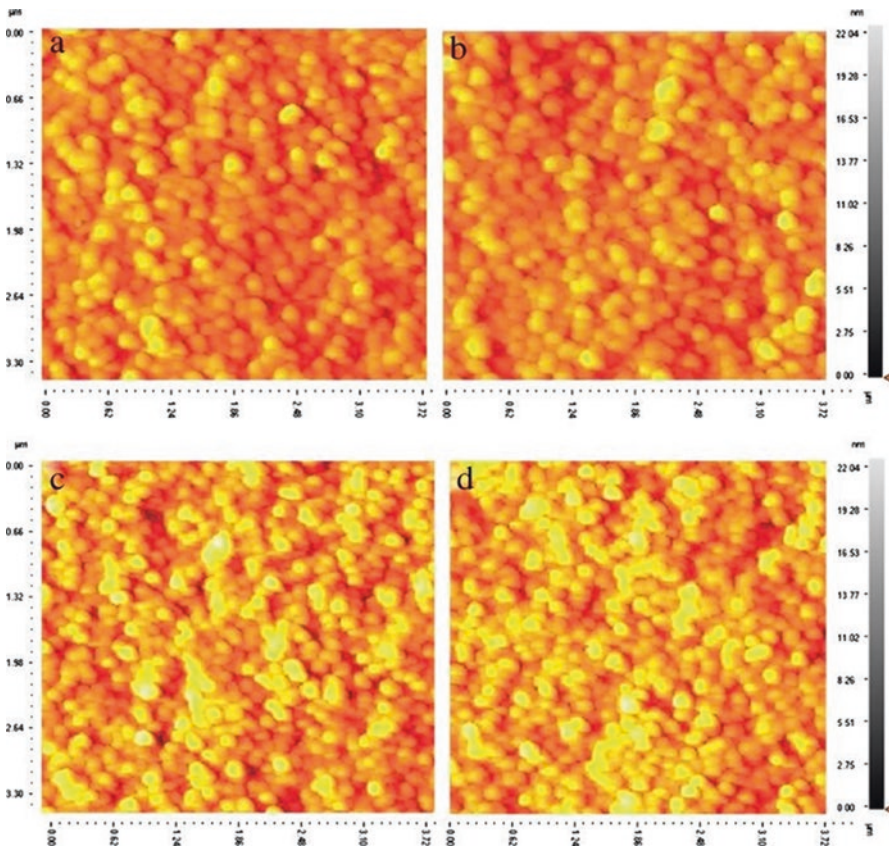
der Waals repulsion, electrostatic interaction and attraction between atoms at the tip and sample surface. The oscillating cantilever scans peaks and valleys over the sample surface in a vertical position and generates a topographical image of up to around 0.5 nm in vertical resolution [4]. During scanning of sample surface, the tip oscillates vertically and contacts the surface alternatively and lifts off, usually at a frequency of 50,000-5,00,000 cycles/s [1].

AFM has different scanning modes, includes dynamic/tapping (contact and intermittent contact with the sample) and static (noncontact) which provides details of various sample parameters like morphological information (size and shape) and elasticity parameters (Young's modulus, adhesion, and stretching) [29] (Fig. 6). However, the major limitation associated with AFM analysis is that the size of the cantilever tip and its geometry is larger than the dimensions of nanoparticles which leads to the widening of the lateral dimensions, which may leads to overestimation of size. AFM has several advantages over the SEM/TEM, which provides a two-dimensional projection of a sample; the AFM provides a three-dimensional surface profile. AFM is capable of producing a three-dimensional topography using just a single scan. In addition, samples viewed by AFM do not require any special treatments (such as metal/carbon coatings) that would damage the nanomaterials. AFM also provides a greater level of detail for particle surfaces, as SEM is not as efficient in resolving the subtle changes on a highly smooth surface. As shown in Fig. 7, Cetin et al. synthesized Eudragit® L100/poly(lactic-co-glycolic acid) (PLGA) based nanoparticles and determined its size and morphology by AFM [30].





**Fig. 6** Schematc diagram of Atomic Force Microscope (AFM)

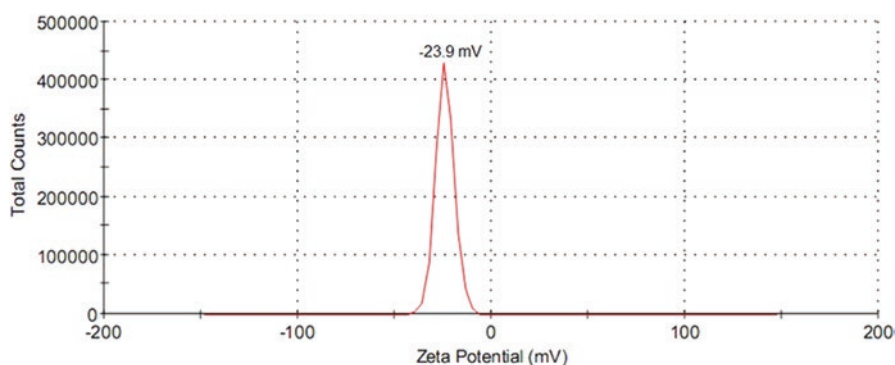


**Fig. 7** AFM images of nanoparticles having various polymer ratio of Eudragit and PLGA (a) 20:80, (b) 30:70, (c) 50:50, and (d) Pure Eudragit (Reprinted with permission from ref. [30])

## 3.2 Surface Charge Analysis

### 3.2.1 Zeta Potential Measurement

As we have discussed previously, measurement of zeta potential is essential to know the stability of nanoparticles. It can be measured by various techniques, like electrophoretic light scattering, acoustic and electro-acoustic. Among these techniques, electrophoretic light scattering is frequently used because of its accuracy, sensitivity and versatility. Moreover, it can simultaneously determine the velocities of many charged species in the sample. The classical electrophoretic light scattering transmits light and receives at a small scattering angle (typically  $8\text{--}30^\circ$ ). It is generally determined by measuring the velocity of the charged molecules towards the electrode in the sample solution due to the presence of an external electric field, which is proportional to the  $\zeta$  potential, and the electrophoretic mobility of nanoparticles is measured by laser Doppler velocimetry (LDV). However, this technique is not suitable for turbid samples because the incident light cannot penetrate the samples. Zeta potential is not only dependent on the surface charge of the nanoparticles but also affected by their surrounding environment like ionic strength, temperature, pH, radiation, nature of the surface ligands and types of ions in the suspension. Therefore, in some cases, when we measure the zeta potential of suspended particles after dilution to produce high resolution and accurate results, it may differ greatly from their original values in that particular environment and may mislead the user [16, 31, 32]. Usually, higher zeta potential value  $> \pm 30$  mV (strongly anionic or cationic) is chosen to infer the particle is stable, whereas a lower value of zeta potential  $< \pm 30$  mV indicates a condition towards aggregation, instability, coagulation or flocculation. Nanoparticles with a zeta potential of  $-10$  mV to  $+10$  mV are considered as neutral [17, 31]. Ngo et al. synthesized gold nanoparticle using of citrate as reducing and stabilising agent and obtained the higher negative zeta potential value  $-23.9$  mV which shows the higher stability of gold nanoparticles [33] (Fig. 8).



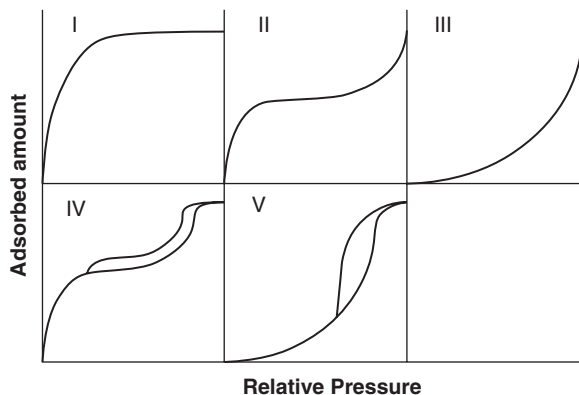
**Fig. 8** Zeta potential graph of synthesized gold nanoparticles (Reprinted with permission from ref. [33])

### 3.3 Surface Area and Porosity

#### 3.3.1 Brunauer, Emmett, and Teller (BET) Analysis

Surface area and porosity are two important physical properties that influence the quality of nanomedicine. The specific surface area of the nanoparticles is the summation of the exposed areas per unit mass. Particle size has inverse relationship with surface area. Several unique properties of nanoparticles are due to their large surface-to-volume ratio. Surface area and porosity properties are also relatable to efficacy and toxicity of nanoparticles. It is therefore important to precisely measure surface area for nano-material characterization. The method of Brunauer, Emmett, and Teller (BET) is used to evaluate the total surface area of nanomaterials. The BET theory assesses the gas-adsorption data and creates aspecific-surface area results which are then expressed in units of area per mass of sample ( $\text{m}^2/\text{g}$ ). The actual surface area including surface pores cannot be estimated from particle size and shape information. Rather, it is determined at the atomic level by the adsorption of an inert gas. Nitrogen adsorption is commonly used to measure the specific surface area of particles. Amount of nitrogen adsorbed not only depends upon exposed surface but also on the temperature, gas pressure and strength of interaction between the gas and solid. Generally, interaction between gas and solid surface is low, the surface need to be cooled using liquid  $\text{N}_2$  to have sufficient detectable quantities of adsorption. As adsorption layers are formed, the sample is taken out from the nitrogen atmosphere and heated to release the adsorbed nitrogen from the nanomaterial and quantified. The data is presented in the form of BET isotherms, which plots the quantity of gas adsorbed on material vs relative pressure. These isotherms may be of different shapes depending upon adsorbent, adsorbate and their interaction. Generally, five types of adsorption-isotherms are used. Type I shows monolayer adsorption and easily explained using Langmuir adsorption theory. This type of isotherm is characteristics of microporous material (pore diameters less than 2 nm) having relatively small external surfaces. Materials like charcoal, molecular sieve zeolites, Metallic Organic Framework (MOFs) and some porous oxides exhibits this type of isotherm. Type II isotherm represents unrestricted monolayer-multilayer adsorption which is characteristics of non-porous or macroporous material like Iron (Fe) catalyst and silica gel. The midway flat region of the isotherm represent the monolayer formation. Type III isotherm explains the formation of unrestricted multilayer. Here lateral interactions between gas molecules are strong compared the interactions between the material surface and adsorbate. Such materials including iodine, bromine, etc. Adsorption on mesoporous materials continues with multilayer adsorption followed by capillary condensation. Mesoporous materials with pore size ranging 2–50 nm, gives type-IV of isotherm. It displays the formation of a monolayer after development of multilayers. Type V isotherms are very similar to type IV isotherms but have relatively weak adsorbate-adsorbent interaction [34] (Fig. 9).

**Fig. 9** Graphical Forms of different BET Isotherms



### 3.4 Chemical Composition and Crystal Structure Analysis

#### 3.4.1 Mass Spectrometry (MS)

Mass spectrometry (MS) is one of the major analytical method which can analyze the samples based on their mass to charge ratio and provides information like mass, chemical composition and elemental composition of a particle or a molecule. MS has high detection sensitivity ( $10^{-9}$  to  $10^{-21}$  mol of sample requires) with high degree of precision and accuracy in determination of molecular weight. Various physico-chemical characteristics of nanoparticles, such as mass, structure and composition can be examined by using different MS procedures and differentiated by their ion sources, separation techniques and detector systems. Among the ionization methods coupled with MS analyzers, electrospray ionization (ESI, usually in conjunction with HPLC/UPLC) and matrix assisted laser desorption/ionization (MALDI) are most frequently used to ionize and volatilize temperature sensitive biomolecules instead of introducing significant decomposition or fragmentation of the molecules. In ESI mode of MS, ions are formed through electrospray by applying a voltage (positive or negative) to liquid flow, which nebulizes the liquid into fine droplets. The droplets travel in high pressure and temperature through the ion source of MS which desolvate droplets and finally release the ions into the gas phase. MALDI-coupled with time of flight-MS (MALDI-TOF)-MS is a highly sensitive and powerful soft ionization technique which is suitable for analysis of complex molecules, like functionalized nanoparticles and proteins [35]. It is a solid phase ionization technique, in which sample and matrix co-crystallized on a solid support. The irradiation from nitrogen laser at 337 nm sublimates the sample/matrix mixture to gas phase where ionization of the sample occurs and proton transfer takes place. The data can be characterized by relatively simple spectra with a pseudomolecular ion,  $[M + H]^+$  for singly charged ions and  $[2M + H]^+$  dimer for doubly charged +2 ion (31). Fig. 10 represents the (MALDI-TOF)-MS spectrum with predictable molecular weight and purity of the synthetic Magainin-I analog peptide (MIAP) [36]. (MALDI-TOF)-MS is useful in the characterization of nanomaterial bioconjugates,

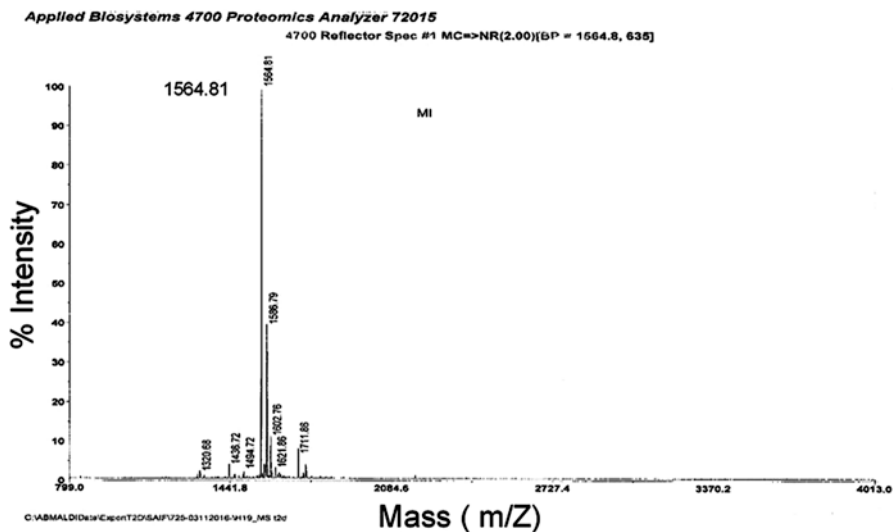


Fig. 10 MALDI-TOF mass spectrum of the synthetic MIAP peptide (Reprinted with permission from ref. [36])

especially in protein based nanoparticles, such as viral nanoparticles, in that mass increases in the viral coat protein because of the addition of biotin or fluorophore species. It is also useful for determination of size/size distributions of nanomaterials, molecular weights of macromolecules, dendrimers and polymers, as well as to illustrate proteins binding to nanoparticles (4, 27). On the other hand, inductively coupled plasma (ICP) ionization MS (ICP-MS) is mainly useful in the analysis of metal containing nanoparticles. It is implemented to validate the conjugation reaction between functionalized nanoparticles and modified contrast agent, where the secondary ion MS provides the molecular and elemental properties of the top layer of nanoparticles, as well as to determine biomaterial surface properties in physiological conditions. However, the application of MS techniques have some limitation in nanomaterials- bioconjugate characterization which may in part because of the relative cost of the instrumentation, the destruction of the sample during measurement, and the required level of expertise needed to run analysis (4, 27).

### 3.4.2 X-Ray Diffraction (XRD)

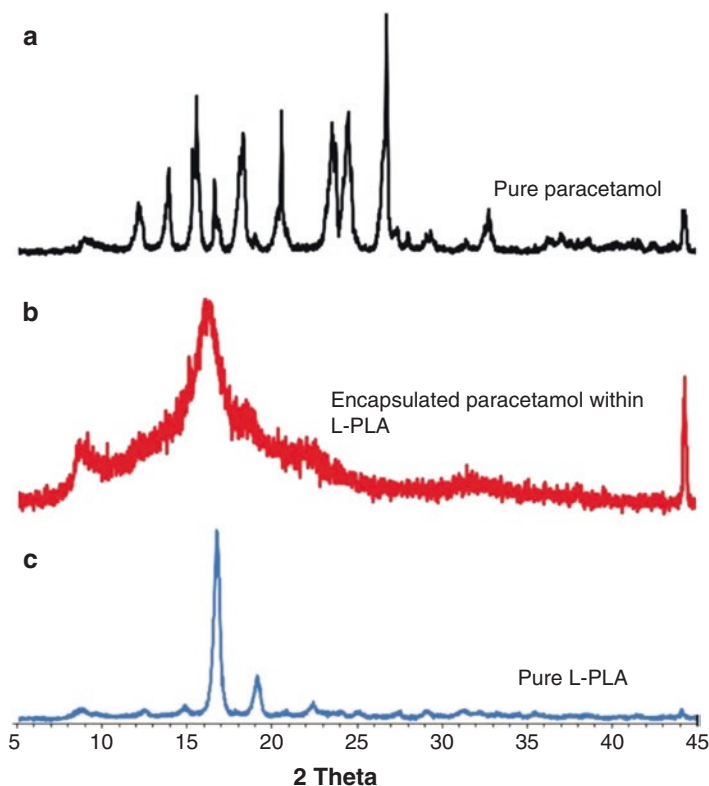
X-ray diffraction (XRD) is a common and effective technique for the study of nanomaterials. The wavelength of X-rays is in atomic scale, hence XRD is an important method for investigating structure of nanoparticles. It helps in completely deciding the tertiary structures of crystalline materials at the atomic scale. Crystalline phases are identified by comparing the interplanar distance values obtained from data. X-rays are electromagnetic radiation with a very short wavelength (few Angstrom)

which is produced when the electrically charged particles with sufficient energy are decelerated. X - rays, generated from cathode ray tube converge as monochromatic collimated radiation and directed towards the sample. The X – rays interfere constructively and destructively producing a diffraction pattern on the detector. Crystalline and semi crystalline materials like polymers, metal, metal oxides nanoparticles have a characteristic atomic structure which diffracts X-rays in a unique diffraction order or pattern. The X-ray diffraction data of polymers or nano-materials gives information regarding crystallinity, orientation of the crystallites, lattice strain, thermal expansion, grain size, internal stress of small crystalline regions, order-disorder transformation, phase composition in semi crystalline polymers and thickness of thin films.

It is also used to determine nanosized components embedded in biological matrix or nanobioconjugate layered materials like nano-hybrids where the analysis of d-spacing alters upon bioconjugation between layers of the nanoparticles. This technique is also helpful to assess the polymorph stability of solid lipid nanoparticles and PEG content on the self assembly of peptide fibril nanostructures [31, 32]. When the pure drug is incorporated in polymer. matrix, change in its crystal property can be measured by XRD. On the basis of different diffraction pattern of pure drug, pure polymer and drug loaded nanoparticles (Fig. 11), incorporation of drug in polymer matrix can be easily distinguished. The major limitation of XRD is its very low diffraction intensity, especially for low atomic number molecules. A recent XRD study shows a novel approach by use of femtosecond pulses generated from a hard X-ray free electron laser for determination of structure of macromolecules which do not have sufficient crystal size [4].

### 3.4.3 Small Angle X-Ray Scattering (SAXS)

In comparison to X-ray diffraction, where applications are confined to crystalline materials, small angle X-ray (SAXS) scattering gives minute detail of different characteristics by determining either amorphous or crystalline materials from polymers, protein to nanoparticles [4]. The principles of SAXS is that, a collision between an incident X-ray beam and a surface particle results elastical-scattering from the sample and forms pattern on a 2D flat X-ray detector which is perpendicular to the direction of the incoming X-ray beam. The reflected waves interfere with each other by constructive interferences at a certain angle and form a peak. The incident X-ray beam interacts with the surface of particles electron clouds and forms scattering pattern according to inhomogeneity in the electron density [4, 26]. By examining the intensity of the scattered X-ray obtained within the scattering angle from 0.1 to 3°, SAXS can determine the size/size distribution, orientation, shape, morphology, structure, and characteristic intra-assembly of a various polymers and nanoparticles in solid/solution form [31, 32]. The periodic variations in the intensity profile are inversely proportional to the particle size as well as the intensity profile of monodisperse particles captures the intensity maxima towards the largest



**Fig. 11** XRD pattern of pure paracetamol, pure L-poly(lactic acid) (L-PLA), and encapsulated paracetamol inside L-PLA (Reprinted with permission from ref. [33])

extent, whereas the smeared intensity minima indicates a modest polydispersity in SAXS data of particle size distribution of gold nanoparticles [37] (Fig. 12).

The recent advancement in SAXS can achieve higher resolution measurements through using synchrotron as the high energy X-ray source.

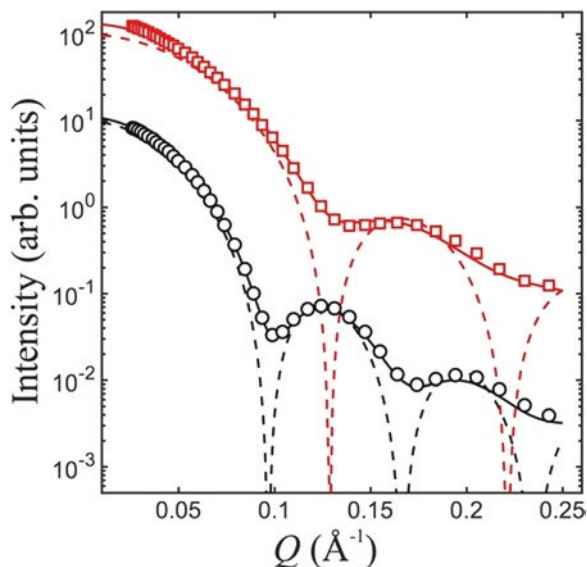
### 3.5 Drug-Polymer Interaction Studies

#### 3.5.1 Differential Scanning Calorimetry (DSC) & Thermogravimetric Analysis (TGA)

Thermal techniques are mainly important in determination of drug-polymer interaction and biomolecules conjugation with nanomaterials and their thermal stability. DSC records the heat released by a chemical process (either a conformational alteration or a chemical reaction) from the test and control samples which are placed in separate chamber of a calorimeter. The heat of reaction ( $\Delta H$ ) that defined as the

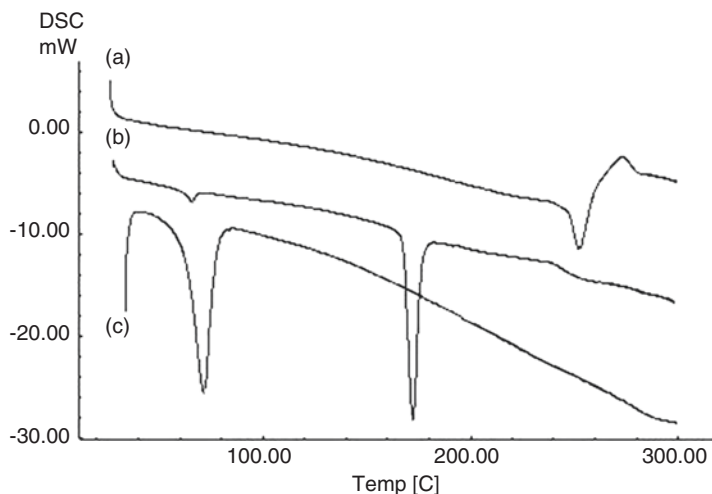


**Fig. 12** 1D SAXS data of suspended gold nanoparticles. Red squares and black circles represent the data from gold nanoparticles of diameter 5 and 10 nm, respectively. The dashed lines and solid lines are intensity profiles from monodisperse and polydisperse spheres, respectively (Reprinted with permission from ref. [34])



change in enthalpy associated with a chemical reaction is recorded by DSC. The positive value of  $\Delta rH$  indicates endothermic reaction, whereas the negative value of  $\Delta rH$  indicates an exothermic reaction. DSC is very useful for measurement of various material transitions including crystallization, melting, decomposition and glass transition. Further analysis can show the state of the nanoparticles-bioconjugate including stability of the biomolecules, underlying crystallinity and interaction of each component with each other. It also helps to elucidate the stability and structure of surface coatings of the nanoparticles-bioconjugate as well as the state of their therapeutic payloads [31]. On the basis of surface area and intensity of endothermal and exothermal peak, the percentage of crystallinity in the drug and polymer can be differentiated. When the drug is incorporated in polymer, it forms molecular dispersion or solid solution in the polymer matrix (Fig. 13). DSC spectra of drug shows broad and weak endotherm that shows transformation of crystalline to amorphous [38].

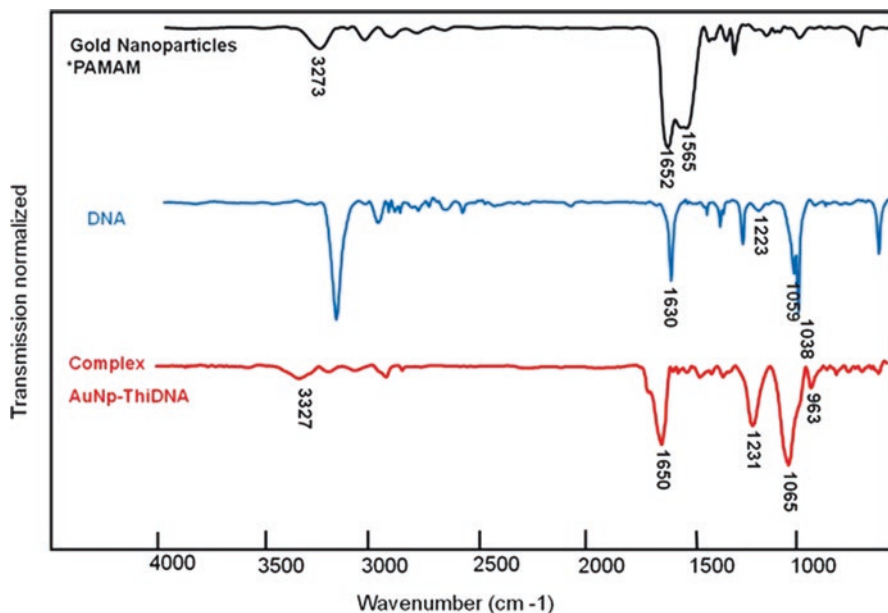
Thermal gravimetric analysis (TGA) is also useful in characterization of thermal stability of compounds. It measures exothermic and endothermic weight loss upon heating and cooling of the nanoparticles and generates its thermal profile. It uses a high precision balance to measure changes in the weight of a sample relative to change in temperature. It characterizes various nanoparticles functionalized with biomolecules on the basis of its unique sequence from physicochemical reactions happening over particular temperature range. Isothermal titration calorimetry is another thermally based technique which can give details about the nanoparticles-bioconjugation. It has potential to determine the affinity, enthalpy and stoichiometry of the nanoparticles-biomolecules interaction [31].



**Fig. 13** DSC thermogram of (A) carboplatin, (B) carboplatin-loaded PCL nanoparticles and (C) PCL polymer (Reprinted with permission from ref. [38])

### 3.5.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR measures the particular absorption of IR radiation, which occurs due to vibrational stretching, and bending of the sample molecules. If the molecules have time-variant dipole moment, their oscillating frequencies are same to the incident light frequency and absorb that frequency of IR radiation. When the molecule absorbs IR radiation, it transfers energy and induces corresponding covalent bond stretching, twisting and bending. Molecules without dipole moments are not absorbing IR radiation, like diatomic molecules of O<sub>2</sub> and N<sub>2</sub>. Normally, vibration in molecules involves a variety of covalent bonds and coupled pairs of atoms and all of them must be considered as a combination of the normal modes, hence, the IR spectra illustrates the absorption or transmission versus incoming IR frequency. It is most frequently used for determination of conjugation between peptide or protein with nanoparticles. In globular proteins, stretching and bending vibrations in the amide region gives secondary structural information which is correspond to the conformational state of the bound protein [31]. The alteration in FTIR spectrum of pure DNA, gold nanoparticles without functionalizations and DNA-gold nanoparticles conjugates indicates the interaction between DNA and gold nanoparticles (Fig. 14). The recently developed attenuated total reflection-FTIR (ATR-FTIR) spectroscopy utilises the total reflection property in conjunction with IR spectroscopy to determine the structural information of adsorbed/deposited molecules at a solid/liquid or solid/air interface, by averting the drawbacks of spectral irreproducibility and sample preparation complexity. ATR-FTIR spectroscopy can be implemented for the analysis of surface features of nanoparticles, although at nanometer scale, it is not a very sensitive surface analysis method as the penetration depth is in the same order of magnitude as the incident IR wavelength [4].



**Fig. 14** FTIR spectra of pure DNA, gold nanoparticles without functionalization and AuNP-DNA nanocomplexes (Reprinted with permission from ref. [39])

### 3.5.3 Ultraviolet-Visible Spectroscopy (UV-Vis)

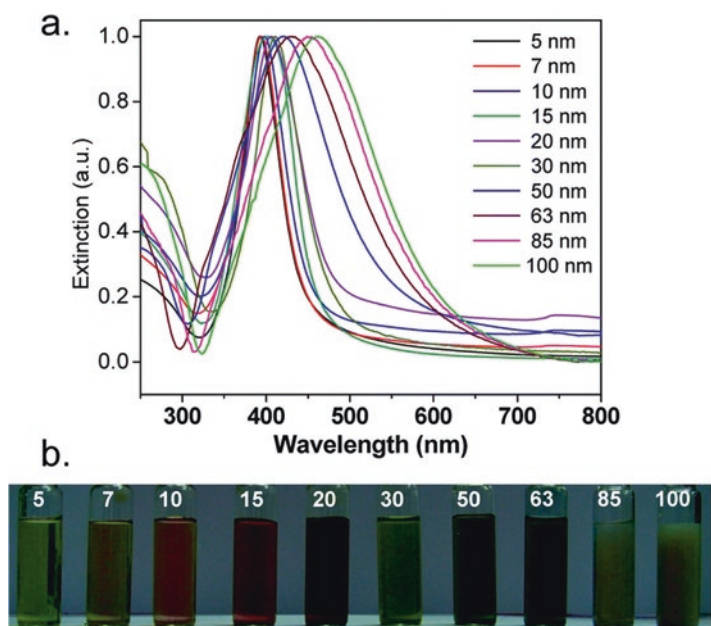
The UV-Vis absorbance of nanoparticles is useful in monitoring its pertinent properties, like size, concentration and aggregation state. Quantum dots have a size-dependent absorption profile which is helpful to characterize its size, composition, and purity. Metal nanoparticles like silver or gold with 40–100 nm size can scatter optical light with remarkable efficiency due to collective resonance of the conduction electrons and shows a strong absorption in the visible region, which is known as the surface plasmon resonance (SPR) band. The adsorption of peptides/protein on the surface of nanoparticles causes some alteration in the absorption spectrum, leading to broadening or shifting of the absorption peak. In case of metal nanoparticles, the alteration in plasmonic peak during peptide/protein adsorption can be monitored [40]. It is affected by various factors, such as size, shape, aggregation state, composition, and refractive index changes within the surface proximity. The wavelength of a light wave and its energy is inversely proportional, so as an increase of nanoparticles size, it absorbs radiation of lower energy.

When the size of gold nanoparticles rises from 10 to 100 nm, the absorption maxima increases from 400 to >560 nm with broadening of the peak. In case of silver nanoparticles, when the silver content increases, the absorption maxima shift towards higher wavelength. It also shows shape dependent peak shift in the spectrum, like pentagon form of particles appear green, the triangular shaped particles appear red, and the spherical particles appear blue. It also shows increase in the

UV-Vis extinction value when the particles size increases from 5–100 nm. Smaller nanospheres primarily absorb light and have peaks near 400 nm, while larger spheres exhibit increased scattering and have peaks that broaden and shift towards longer wavelengths (known as red-shifting). Shape dependent peak shift in the spectrum, like pentagon form of particles appear green, the triangular shaped particles appear red, and the spherical particles appear blue. It also shows increase in the UV-Vis extinction value (Fig. 15) when the particles size increases from 5–100 nm [41]. Smaller nanospheres primarily absorb light and have peaks near 400 nm, while larger spheres exhibit increased scattering and have peaks that broaden and shift towards longer wavelengths (known as red-shifting).

### 3.6 Stability of Drug Nanoparticles

The high surface area to volume ratio of nanoparticles may cause the reactive and colloidal instability as compared to their bulk. In general, nanoformulation stability is categorized in to physical, chemical and pharmaceutical stability. The common physical stability issues with nanoparticle formulations include agglomeration, sedimentation/ creaming, crystal growth and change of crystallinity state. The selection



**Fig. 15** (a) UV-Vis extinction spectrum and (b) the distinctive color of 5–100 nm sized silver nanoparticles (Reprinted with permission from ref. [41])

of characterization methodology for nanoparticles- stability is dependent on the kind of stability issues and formulations.

### **3.6.1 Sedimentation/ Creaming**

The changes in nanoparticle size is usually used to predict the stability of most of the nanomedicine. The deviations from the average size range is an indication of nanoparticle association or dissociation or instability in that specific environment. Sometimes, nanoparticles can settle down in the medium depending on their density comparative to the medium. Decreasing particle size is the most common strategy used to reduce particle settling. Large particles (microscale or more) precipitate more easily due to gravitational force, whereas nanoscale particles below one micron do not settle due to Brownian motion. Conventional method of evaluation of sedimentation is visual observation over a span of time. The quantitative volume of sedimentation is evaluated by measuring settled volume relative to the total suspension volume in specific time. Using dynamic light scattering we illustrate that cluster size and fractal dimension which should be considered when evaluating the fate of aggregated nanomaterials.

### **3.6.2 Agglomeration**

Aggregation of nanoparticles is serious issue which disrupt various properties of nanoformulations and leading to destabilization of colloidal systems. Aggregation depends on the type of nanomaterial, reagents or method used for nanoparticle synthesis. In this process, nanoparticles dispersed in the aqueous phase stick to each other to form asymmetrical clusters, flocs, or aggregates. It modifies the physicochemical properties, activity, transport and biological interactions of nanoparticles. The unique properties of nanoparticles due to their size significantly changes due to aggregation. A quantitative measurement of nanoparticles aggregation would deliver a valuable assessment of colloidal stability. DLS is a very powerful characterization technique as it yields absolute values for an ensemble of particles. The variations of the intensity of light scattered by a nanoparticle dispersion are observed over a period of time and the analysis give yields information about the hydrodynamic radius (R) of the sample. Cryogenic transmission electron microscopy (cryo-TEM) is also used for the evaluation agglomeration of nanoparticles. Here sample is investigated under frozen-hydrated conditions which includes plunge freezing of aqueous sample. Another technique which is used to evaluate the agglomeration is Asymmetric flow field-flow fractionation (AF-FFF). This is a separation technique based on the theory of field flow fractionation (FFF) which is usually used for sample separation and size characterization of nanoparticles both in aqueous as well as organic solution. The separation is attained by cross-flow of suspension of nanoparticles in a narrow, ribbon-like channel which is built up by a spacer, between a porous and a nonporous plate. The porous plate is covered by a membrane allows

the liquid to pass the membrane, retain the nanoparticles. Extensive characterization of nanoparticles and their aggregates is possible by coupling AF-FFF with online detectors like UV, fluorescence detector etc.

### 3.6.3 Shelf Life

The self-life of the nano-medicine depends upon their chemistry, morphology and storage conditions. Depending upon the chemistry, polymer absorb moisture on storage which initiate degradation and a change in physicochemical properties, which in turn can alter their *in-vivo* performance. The presence of residual solvent, residual monomer or catalysts may weaken the storage stability and leading to degradation. The relative strength of water-polymer bonds and the process of crystallization also affects degradation of nanomedicine. The storage of nanomedicine is recommended in an inert environment to maintain physicochemical integrity of nanomedicine. Additionally, drug leakage, degradation and microbiological growth can be other issues that can cause degradation of nano-medicine. HPLC and LC-MS are the most common method used to assess the chemical stability which gives detailed quantitative analysis of degradation impurities. MS usually coupled with LC-MS or HPLC are used to ascertain the molecular structure of impurities. Some other techniques such as Fourier-transform infrared spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR) can also be used for chemical stability assessment Table 2.

## 4 Regulatory Requirement

Nano-medicines are complex products which are the result of difficult to control manufacturing processes. Detection and characterization of nanomaterials in complex matrices was considered an important issue by the regulatory community. Credible characterization methods for nanoparticles will significantly affect the uptake of these nanomaterial in commercial applications and allow the industry to comply with regulation. The identification of various critical points of nano-

**Table 2** A few commonly used stability characterization techniques are listed

Parameters	Techniques
Sedimentation/creaming	Visual observation/laser backscattering/near infrared transmission
Agglomeration	DLS/Cryo-TEM/ Asymmetric flow field-flow fractionation
Chemical stability	HPLC/FTIR/NMR/MS
Shape	SEM/AFM/TEM

material products in existing legal framework to re-evaluate the changing characteristic properties is necessary requirement. There are several challenges in the characterization of nano-materials because of the interdisciplinary nature, the absence of suitable reference materials for the calibration, the difficulties linked to the sample preparation for analysis and the interpretation of the data. To correct for this, we need important standard methods to characterize available nanoscale reference materials (RMs) demonstrating their relevance for the characterization of nanomedicines. The United States-Nanotechnology Characterization Laboratory (US NCL) and the European-Nanotechnology Characterization Laboratory (EU-NCL) have developed and optimized protocols for the physicochemical and biological characterization of candidate nanomedicines. The International Organization for Standardization (ISO) and American Society for Testing and Materials (ASTM) International also have developed and published several standardized test methods, guidance, and reports dedicated to the physicochemical and biological characterization of engineered nanomaterials. It compiles general informative documents and guidelines offering an overview of existing methods to determine basic physicochemical and toxicological characteristics of nanomaterials. These documents highlight the relevance and the limitations of different techniques and include special considerations for testing of nanomaterials. The guidance covers all aspects of testing including nanomaterial characterization, sample preparation, toxicological evaluation, and risk assessment considerations.

## 5 Conclusion

The physicochemical characteristics at the nanoscale have the potential to influence physiological interactions from the molecular level to the physiological level. The rapid development and manufacture of nanoparticles for the use as drug carrier systems needs appropriate regulations. The measurement and characterization of nanomedicine poses several analytical challenges for scientists, developers, and regulatory agencies. Several practical guidelines for the characterization and quality control of nanoformulations are needed. Appropriate robust techniques for nanoparticles characterization are essential to ensure regulatory guidelines for efficacy and safety of nanomedicines. This chapter describes the important physicochemical properties of nanoparticles, followed by general overview to various methods, which are commonly used for characterizing nanoparticles. The short description of each technique together with their range of applications in nanomaterial characterization is described.



## References

1. Joshi, M.; Bhattacharyya, A.; Ali, S. W. Characterization techniques for nanotechnology applications in textiles. 2008.
2. Boegh M, Foged C, Müllertz A, Nielsen HM. Mucosal drug delivery: barriers, in vitro models and formulation strategies. *Journal of Drug Delivery Science and Technology*. 2013;23(4):383–91.
3. Ong KJ, MacCormack TJ, Clark RJ, Ede JD, Ortega VA, Felix LC, Dang MK, Ma G, Fenniri H, Veinot JG, Goss GG. Widespread nanoparticle-assay interference: implications for nanotoxicity testing. *PLoS One*. 2014;9(3):e90650. <https://doi.org/10.1371/journal.pone.0090650>.
4. Lin P-C, Lin S, Wang PC, Sridhar R. Techniques for physicochemical characterization of nanomaterials. *Biotechnol Adv*. 2014;32(4):711–26.
5. Laffleur F, Bernkop-Schnürch A. Strategies for improving mucosal drug delivery. *Nanomedicine*. 2013;8(12):2061–75.
6. Lu, Y.; Park, K. Appendix f: chapter ii. 5.16—drug delivery systems: H, Mucosal drug delivery.
7. Hoshyar N, Gray S, Han H, Bao G. The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. *Nanomedicine*. 2016;11(6):673–92. <https://doi.org/10.2217/nmm.16.5>.
8. Desai MP, Labhasetwar V, Walter E, Levy RJ, Amidon GL. The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent. *Pharm Res*. 1997;14(11):1568–73. <https://doi.org/10.1023/a:1012126301290>.
9. Gaumet M, Vargas A, Gurny R, Delie F. Nanoparticles for drug delivery: the need for precision in reporting particle size parameters. *Eur J Pharm Biopharm*. 2008;69(1):1–9.
10. Patri A, Dobrovolskaia M, Stern S, McNeil S, Amiji M. Preclinical characterization of engineered nanoparticles intended for cancer therapeutics. *Nanotechnology for cancer therapy*. 2007:105–38.
11. Norris DA, Puri N, Sinko PJ. The effect of physical barriers and properties on the oral absorption of particulates. *Adv Drug Deliv Rev*. 1998;34(2–3):135–54.
12. Lai SK, Wang YY, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev*. 2009;61(2):158–71. <https://doi.org/10.1016/j.addr.2008.11.002>.
13. Powers KW, Brown SC, Krishna VB, Wasdo SC, Moudgil BM, Roberts SM. Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. *Toxicol Sci*. 2006;90(2):296–303.
14. Toy R, Peiris PM, Ghaghada KB, Karathanasis E. Shaping cancer nanomedicine: the effect of particle shape on the in vivo journey of nanoparticles. *Nanomedicine*. 2014;9(1):121–34. <https://doi.org/10.2217/nmm.13.191>.
15. Yu MK, Park J, Jon S. Targeting strategies for multifunctional nanoparticles in cancer imaging and therapy. *Theranostics*. 2012;2(1):3–44. <https://doi.org/10.7150/thno.3463>.
16. Xu R. Progress in nanoparticles characterization: sizing and zeta potential measurement. *Particuology*. 2008;6(2):112–5.
17. Clogston JD, Patri AK. Zeta potential measurement. In: *Characterization of nanoparticles intended for drug delivery*: Springer; 2011. p. 63–70.
18. Bhatia S. Nanoparticles types, classification, characterization, fabrication methods and drug delivery applications. In: *Natural polymer drug delivery systems*: Springer; 2016. p. 33–93.
19. Gabor F, Bogner E, Weissenboeck A, Wirth M. The lectin-cell interaction and its implications to intestinal lectin-mediated drug delivery. *Adv Drug Deliv Rev*. 2004;56(4):459–80. <https://doi.org/10.1016/j.addr.2003.10.015>.
20. Menzel C, Bernkop-Schnürch A. Enzyme decorated drug carriers: targeted swords to cleave and overcome the mucus barrier. *Adv Drug Deliv Rev*. 2018;124:164–74. <https://doi.org/10.1016/j.addr.2017.10.004>.
21. Soppimath KS, Aminabhavi TM, Kulkarni AR, Rudzinski WE. Biodegradable polymeric nanoparticles as drug delivery devices. *J Control Release*. 2001;70(1–2):1–20.

22. Cho EJ, Holback H, Liu KC, Abouelmagd SA, Park J, Yeo Y. Nanoparticle characterization: state of the art, challenges, and emerging technologies. *Mol Pharm.* 2013;10(6):2093–110.
23. Berne BJ, Pecora R. *Dynamic light scattering: with applications to chemistry, biology, and physics*: Courier Corporation; 2000.
24. Griffiths, P. C.; Cattoz, B.; Ibrahim, M. S.; Anuonye, J. C. Probing the interaction of nanoparticles with mucin for drug delivery applications using dynamic light scattering. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V* 2015, 97 (Pt A), 218–222, doi: <https://doi.org/10.1016/j.ejpb.2015.05.004>.
25. Mourdikoudis S, Pallares RM, Thanh NTK. Characterization techniques for nanoparticles: comparison and complementarity upon studying nanoparticle properties. *Nanoscale.* 2018;10(27):12871–934. <https://doi.org/10.1039/c8nr02278j>.
26. Singh AK. *Engineered nanoparticles: structure, properties and mechanisms of toxicity*: Academic; 2015.
27. Luo Z. *A practical guide to transmission Electron microscopy, volume II: advanced microscopy*: Momentum Press; 2015.
28. Nagashima, K.; Zheng, J.; Parmiter, D.; Patri, A. K. Biological tissue and cell culture specimen preparation for TEM nanoparticle characterization. In *Characterization of Nanoparticles Intended for Drug Delivery*; Springer: 2011; pp 83–91.
29. Scalf J, West P, Part I. Introduction to nanoparticle characterization with AFM. *Pacific Nanotechnology: Santa Clara* (see [www.nanoparticles.org/pdf/Scalf-West.pdf](http://www.nanoparticles.org/pdf/Scalf-West.pdf)); 2006.
30. Cetin M, Atila A, Kadioglu Y. Formulation and in vitro characterization of Eudragit® L100 and Eudragit® L100-PLGA nanoparticles containing diclofenac sodium. *AAPS PharmSciTech.* 2010;11(3):1250–6.
31. Sapsford KE, Tyner KM, Dair BJ, Deschamps JR, Medintz IL. Analyzing nanomaterial bioconjugates: a review of current and emerging purification and characterization techniques. *Anal Chem.* 2011;83(12):4453–88.
32. Ghosh Chaudhuri R, Paria S. Core/shell nanoparticles: classes, properties, synthesis mechanisms, characterization, and applications. *Chem Rev.* 2011;112(4):2373–433.
33. Ngo VKT, Nguyen HPU, Huynh TP, Tran NNP, Lam QV, Huynh TD. Preparation of gold nanoparticles by microwave heating and application of spectroscopy to study conjugate of gold nanoparticles with antibody E. coli O157: H7. *Adv Nat Sci Nanosci Nanotechnol.* 2015;6(3):035015.
34. Naderi M. Chapter Fourteen - Surface Area: Brunauer–Emmett–Teller (BET). In: *Progress in Filtration and Separation*: Academic; 2015. p. 585–608.
35. Ramalinga U, Clogston JD, Patri AK, Simpson JT. Characterization of nanoparticles by matrix assisted laser desorption ionization time-of-flight mass spectrometry. In: *Characterization of nanoparticles intended for drug delivery*: Springer; 2011. p. 53–61.
36. Sharma A, Vaghasiya K, Gupta P, Gupta UD, Verma RK. Reclaiming hijacked phagosomes: hybrid nano-in-micro encapsulated MIAP peptide ensures host directed therapy by specifically augmenting phagosome-maturation and apoptosis in TB infected macrophage cells. *Int J Pharm.* 2018;536(1):50–62.
37. Wang W, Zhang H, Kuzmenko I, Mallapragada S, Vaknin D. Assembling bare au nanoparticles at positively charged templates. *Sci Rep.* 2016;6:26462.
38. Alex AT, Joseph A, Shavi G, Rao JV, Udupa N. Development and evaluation of carboplatin-loaded PCL nanoparticles for intranasal delivery. *Drug Deliv.* 2016;23(7):2144–53.
39. Rolim TI, Cancino J, Zucolotto V. A nanostructured genosensor for the early diagnosis of systemic arterial hypertension. *Biomed Microdevices.* 2015 Feb;17(1):3.
40. Rahman M, Laurent S, Tawil N, Yahia LH, Mahmoudi M. Analytical methods for corona evaluations. In: *Protein-Nanoparticle Interactions*: Springer; 2013. p. 65–82.
41. Agnihotri S, Mukherji S, Mukherji S. Size-controlled silver nanoparticles synthesized over the range 5–100 nm using the same protocol and their antibacterial efficacy. *RSC Adv.* 2014;4(8):3974–83.