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Research review paper

Techniques for physicochemical characterization of nanomaterials

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ABSTRACT

Advances in nanotechnology have opened up a new era of diagnosis, prevention and treatment of diseases and traumatic injuries. Nanomaterials, including those with potential for clinical applications, possess novel physicochemical properties that have an impact on their physiological interactions, from the molecular level to the systemic level. There is a lack of standardized methodologies or regulatory protocols for detection or characterization of nanomaterials. This review summarizes the techniques that are commonly used to study the size, shape, surface properties, composition, purity and stability of nanomaterials, along with their advantages and disadvantages. At present there are no FDA guidelines that have been developed specifically for nanomaterial based formulations for diagnostic or therapeutic use. There is an urgent need for standardized protocols and procedures for the characterization of nanoparticles, especially those that are intended for use as theranostics.

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1. Introduction

The emerging field of nanomedicine utilizes nanomaterials to improve diagnosis, prevention and treatment of diseases (Duncan and Gaspar, 2011). According to the Nanotechnology Characterization Laboratory (NCL) at the National Cancer Institute, National Institutes of Health nanoparticles (NPs) have a size range between 1 and 100 nm (McNeil, 2005). Nanomaterials have at least one dimension in the range of sub-nanometer to 10 nm. Small molecules and certain naturally occurring biological materials are not usually referred to as nanomaterials, even though they may be in the range of 1 to 100 nm. Research on manmade nanomaterials and engineered nanomaterials in the 1 to 100 nm range has gathered momentum because of their potential for a diverse array of applications in science, technology and medicine (Webster, 2006). Some examples of nanomaterials include liposomes, dendrimers, carbon nanorods, carbon nanotubes, fullerenes, graphene derivatives, titanium oxides, gadolinium nitride nanowires, silver NPs, gold NPs, platinum NPs, magnetic NPs and quantum dots (Duncan and Gaspar, 2011; Mahajan et al., in press; Singh and Sahoo, in press; Wong et al., in press).

When a solid is split, it exposes two new surfaces; with every subsequent cut, newer surfaces emerge. As any material is broken down to very small particles, the surface area per unit mass increases dramatically. Nanomaterials are characterized by a relatively large surface area per unit mass. Since the surface area of a solid depends on its shape, *e.g.* a sphere has the smallest surface area per unit mass, the surface area of nanomaterials depends on the size as well as shape. Changes in size or shape of nanomaterials can affect their physicochemical and physiological properties.

The physiological interactions in the body influenced by the biodistribution, passage, phagocytosis and endocytosis of nanomaterials through tissues may differ from those of conventional medicines (Gref et al., 1994). In order to realize the full potential of nanomedicines, it is necessary to develop robust standards for characterizing the engineered/fabricated nanomaterials, for example, to provide a guidance for ensuring quality control and assessing the safety as well as toxicity of nanomaterials (Pleus, 2012). Characteristics such as molecular structure, chemical composition, melting point, boiling point, vapor pressure, flash point, pH, solubility, and water octanol partition coefficient have to be determined for nanomaterials in the same manner as they are for larger non-nanomaterials. In addition, nanomaterial characterization places special emphasis on parameters such as size/size distribution, porosity (pore size), surface area, shape, wettability, zeta potential, adsorption isotherm (adsorption potential), aggregation, distribution of conjugated moieties and impurities.

At present there are no U.S. Food and Drug Administration (FDA) guidelines developed specifically for nanomaterial based formulations for diagnostic or therapeutic use. However, the agency has issued two product-specific draft guidance documents to address the utilization of nanotechnology in the food and cosmetics industries (http://www.fda.gov/ScienceResearch/SpecialTopics/Nanotechnology/ucm301093.htm). This can be a stepping stone towards detection or characterization of nanomaterials, although currently there are no standardized methodologies or regulatory protocols. Still, the NCL, serving as "a national resource and knowledge base" to assist the regulatory review of nanotechnologies and the development and translation of nanoparticles and devices for clinical applications, characterizes the physicochemical properties, *in vitro* biological properties and *in vivo* compatibility of nanoparticles (http://ncl.cancer.gov/about_mission.asp). The assay cascade protocols at the NCL include a number of methods to investigate nanomaterials' characteristics, such as size, molecular weight, aggregation, purity, chemical composition and surface properties. The NCL protocols also include methods for determining sterility, drug release and toxicity *in vitro*, and efficacy, disposition and immunotoxicity *in vivo* (http://ncl.cancer.gov/working_assay-cascade.asp). Similarly, the European Union has formed the unit of Registration, Evaluation, Authorization and Restriction of Chemicals, by which nanomaterials are regulated.

Many methods have been used for evaluating manufactured nanomaterials, including techniques in optical spectroscopy, electron microscopy, surface scanning, light scattering, circular dichroism, magnetic resonance, mass spectrometry, X-ray scattering and spectroscopy, and zeta-potential measurements, as well as methods in the categories of thermal techniques, centrifugation, chromatography, and electrophoresis (Sapsford et al., 2011). In this review article, we briefly describe the principles, applications, strengths and limitations of a variety of modalities commonly used to investigate the physicochemical characteristics of nanomaterials (Table 1).

2. Overview of physicochemical characteristics

Typically, engineered materials with dimensions in the nanometer scale are intermediates between isolated small molecules and bulk materials. Nanomaterials, which are similar to biological moieties in scale, can be used as diagnostic and therapeutic nanomedicines (Del Burgo et al., in press; Hachani et al., 2013; Kim et al., 2010). Compared to their bulk material counterparts, the distinct physicochemical properties of the nanomaterials, such as size, surface properties, shape, composition, molecular weight, identity, purity, stability and solubility, are critically relevant to particular physiological interactions (Table 2) (Patri et al., 2006). These physiological interactions may provide benefits in medical applications, including improvements in efficacy, reduction of side effects, prevention and treatment (Farokhzad and Langer, 2006; Hall et al., 2007).

Impact of nanomaterials on their physiological behaviors will influence the therapeutic efficacy and/or diagnostic accuracy of nanomedicines. In this context, it is important to understand how the different physicochemical characteristics of nanomaterials affect their *in vivo* distribution and behavior. This demands reliable and robust techniques for studying the different physicochemical characteristics of nanomaterials in general and nanomedicines in particular. The different techniques used for characterization of nanomaterials, based on their different features, are described in the following sections. A rigorous but practical approach to reliable characterization of nanomaterials is essential for quality assurance and safe, rational development of nanomedicines and theranostics (Akhter et al., 2013; Kim et al., 2013).

2.1. Size

In engineered nanomaterials, size is a crucial factor that regulates the circulation and navigation of nanomaterials in the bloodstream, penetration across the physiological drug barriers, site- and cell-specific localization and even induction of cellular responses (Feng, 2004; Ferrari, 2008; Jiang et al., 2008). In general, the size of a nonspherical nanomaterial is defined as an equivalent diameter of a spherical particle whose selected physical properties, *e.g.* diffusivity, are equivalent to those of the nanomaterial in the same environment (Powers et al., 2006; Shekunov et al., 2007). One frequently adopted example is the hydrodynamic diameter of a molecule, which is the effective size

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calculated from the diffusion coefficient using the Stokes–Einstein relationship (Powers et al., 2006).

Lately there has been public and government concern about the toxicity of nanomaterials and their related adverse health effects, such as pronounced pulmonary inflammation (Horváth et al., 2013; Karlsson et al., 2009; Oberdörster, 2005). Other examples include the smaller silver NPs causing a greater apoptotic effect against certain cell lines and 20 nm silica NPs exhibiting more toxicity than negatively-charged 100 nm silica NPs (Kim et al., 2012; Park et al., 2013; Sosenkova and Egorova, 2011). Although NPs with certain chemical compositions were reported to be more toxic compared to their larger counterparts of the same composition, a consensus on the increased toxicity and putative health risks of nanomaterials may not emerge due to the lack of obvious size-related change in toxicity in other NPs, *e.g.* titanium oxide and iron oxides (Buzea et al., 2007; Horváth et al., 2013; Karlsson et al., 2009; Park et al., 2007; Warheit et al., 2006). The relationship of size and/or shape to nanoparticle toxicity or nanomedicine efficacy has to be investigated on a case by case basis, because of the wide differences in the behavior of different nanomaterials.

2.2. Surface properties

Many characteristics of nanomaterial interfaces are functions of atomic or molecular compositions of the surfaces and the physical surface structures that respond to the interactions of the nanomaterial with surrounding species (Patri et al., 2006; Powers et al., 2006). From the aspect of nanomedicine, these characteristics are considered the elements of surface properties in the environment of biological fluid (Patri et al., 2006; Powers et al., 2006). Among the different surface properties, surface composition, surface energy, wettability, surface charge and species absorbance or adhesion are commonly considered important parameters (Brodbeck et al., 2001; Patri et al., 2006; Powers et al., 2006; Ratner et al., 2004; Vertegel et al., 2004). Surface composition is intrinsically relevant to the superficial layers but not to the bulk materials. Surface energy is relevant to the dissolution, aggregation and accumulation of nanomaterial. Surface charge, with potential effect on receptor binding and physiological barrier penetration, governs the dispersion stability or aggregation of nanomaterials and is generally estimated by zeta potential. Finally, species absorbance or adhesion potentially alters the surface of nanomaterial as well as the conformation and the activity of the attached species. However, investigation of the entire spectrum of surface parameters is impractical, and prioritization of the surface parameters requires independent validation for each nanomaterial system (Powers et al., 2006; Ratner et al., 2004).

Recent studies have shown improvement of cellular and lysosome uptakes of positively-charged nanomaterials, compared with their neutral or negatively-charged counterparts (Asati et al., 2010; Baoum et al., 2010; Klesing et al., 2010; Liu et al., 2011; Luyts et al., 2013). The enhanced uptake of positively-charged NPs makes them attractive as agents for tumor drug delivery: poly(D,L-lactide-*co*-glycolide)-formulated NPs with cationic chitosan are useful for localized, sustained gene delivery to the alveolar epithelium (Baoum et al., 2010). However, positively-charged nanomaterials can be more toxic than their negatively-charged counterparts. The positively-charged amino-modified polystyrene-formulated NPs were cytotoxic to certain cell lines by inducing DNA damage (Liu et al., 2011). Positively-charged branched polyethyleneimine coated Ag NPs were highly toxic to certain *bacillus* species in which the NPs caused membrane damage (El Badawy et al., 2010). Cytotoxicity of positively-charged Si NP-NH_2 towards macrophage NR8383 cells involved effects on phagocytosis, mitochondrial disruption and the production of high levels of intracellular reactive oxygen species (Bhattacharjee et al., 2010). In contrast, the effects of surface charge on cytotoxicity and reactive oxygen species generation were enhanced in the negatively-charged silica NPs of 20 nm in size, compared with those induced by silica NPs of the same size, but weaker negative charge (Park et al., 2013). Although the connection between increased cellular uptake of positively-charged NPs and elevated cytotoxicity was typically demonstrated in *in vitro* studies, *in vivo* evidence is less convincing (Luyts et al., 2013). The relation between surface charge/zeta potential and NP toxicity cannot be generalized (Luyts et al., 2013).

2.3. Shape

In addition to size and surface properties, the shape of nanomaterial can play an important role in drug delivery, degradation, transport, targeting and internalization (Champion et al., 2007; Decuzzi et al., 2009; Euliss et al., 2006; Geng et al., 2007; Gratton et al., 2008; Jiang et al., 2013; Mitragotri, 2009). Efficiency of drug delivery carriers was highly influenced by controlling the shapes of the carriers (Champion et al., 2007; Decuzzi et al., 2009), while phagocytosis of drug delivery carriers through macrophages was also dependent on carrier shape (Champion and Mitragotri, 2009). Furthermore, flow and adhesion of drug delivery carriers throughout the circulatory system and the *in vivo* circulation time of the nanomedicine can be controlled by modulating the shapes of drug-loaded nanomaterials (Doshi et al., 2010; Geng et al., 2007).

The shape of nanomaterial affects cellular uptake, biocompatibility and retention in tissues and organs (George et al., 2012; Pal et al., 2007). Additionally, the disposition and translocation of nanomaterials in the organism may be influenced by their shape, accompanying size and state of agglomeration (Powers et al., 2009). One example is an *in vitro* study of silica NPs demonstrating shape-driven agglomeration as a potential trigger in the pulmonary pathogenesis (Brown et al., 2007). Another example is the higher toxicity of dendrimer-shaped nickel NPs compared to that of the spherical ones towards zebrafish embryos (Ispas et al., 2009). Similarly, plate-shaped silver NPs were more hazardous than spherical, rod-shaped or wire shaped silver nanoparticles when tested against *Escherichia coli* and zebrafish embryos (George et al., 2012; Pal et al., 2007). Furthermore, recent studies demonstrated an asbestos-like pathogenic response when carbon nanotubes of length greater than 20 μm were delivered into the abdominal cavity of mice (Kostarelos, 2008; Poland et al., 2008; Powers et al., 2009; Takagi et al., 2008).

2.4. Composition and purity

A broad variety of nanomaterials are utilized in the production of approved or potential nanomedicines. These nanomaterials can be categorized by their structural types, such as NP and its derivatives, liposome, micelle, dendrimer/fleximer, virosome, emulsion, quantum dot, fullerene, carbon nanotube and hydrogel, and each type may consist of polymers, metals and metal oxides, lipids, proteins, DNA or other organic compounds (Etheridge et al., 2013; Patri et al., 2006). Composition of a nanomaterial affects transport, delivery and biodistribution. In biomedical applications of nanomaterials, there may be a need to combine two or more types of nanomaterials to form a complex such as a chelate, a conjugant or a capsule. Consequently chemical composition analysis of the nanomaterial complex is more complicated than that for a single entity (Patri et al., 2006).

There are several studies addressing toxicological concerns about NPs of different compositions (Hardman, 2006). In addition to size and shape, chemical composition is another important factor in determining toxicity of NPs (Buzea et al., 2007; Hardman, 2006). For example, TiO_2 induced an inflammatory neutrophil response when intratracheally instilled in rat and mouse lungs (Oberdörster, 2005; Sohaebuddin et al., 2010). In addition, cytotoxicity is generally observed in quantum dots with core metalloid complexes consisting of widely used metals such as cadmium and selenium (Hardman, 2006). Still, quantum dots can be rendered nontoxic, when core coatings are appropriately registered; alternatively, the cytotoxicity of quantum dots was only observed after degradation of their core coating *in vivo* or *in vitro* (Buzea et al., 2007; Derfus et al., 2003; Hardman, 2006).

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Table 1
Analytical modalities for evaluation of the physicochemical characteristics of nanomaterials.

Techniques	Physicochemical characteristics analyzed	Strengths	Limitations	Refs
Dynamic light scattering (DLS)	Hydrodynamic size distribution	Non-destructive/invasive manner Rapid and more reproducible measurement Measures in any liquid media, solvent of interest Hydrodynamic sizes accurately determined for monodisperse samples Modest cost of apparatus	Insensitive correlation of size fractions with a specific composition Influence of small numbers of large particles Limit in polydisperse sample measures Limited size resolution Assumption of spherical shape samples	Brar and Verma (2011); Domingos et al. (2009); Filipe et al. (2010); Murdock et al. (2008); Pan et al. (2013); Sapsford et al. (2011); Schacher et al. (2009); Wagner et al. (2007); Zhao et al. (2013)
Fluorescence correlation spectroscopy (FCS)	Hydrodynamic dimension Binding kinetics	High spatial and temporal resolution Low sample consumption Specificity for fluorescent probes Method for studying chemical kinetics, molecular diffusion, concentration effect, and conformation dynamics	Limit in fluorophore species Limited applications and inaccuracy due to lack of appropriate models	Boukari and Sackett (2008); Domingos et al. (2009); Jing and Zhu (2011); Nienhaus et al. (2013); Sapsford et al. (2011)
Zeta potential	Stability Referring to surface charge	Simultaneous measurement of many particles (using ELS)	Electro-osmotic effect Lack of precise and repeatable measurement	Choi et al. (2011); Clogston and Patri (2011); Khatun et al. (2012); Sapsford et al. (2011); Weiner et al. (1993); Xu (2008)
Raman scattering (RS) Surface enhanced Raman (SERS) Tip-enhanced Raman spectroscopy (TERS)	Hydrodynamic size and size distribution (indirect analysis) Conformation change of protein–metallic NP conjugate Structural, chemical and electronic properties	Complementary data to IR No requirement of sample preparation Potential of detecting tissue abnormality Enhanced RS signal (SERS) Increased spatial resolution (SERS) Topological information of nanomaterials (SERS, TERS)	Relatively weak single compared to Rayleigh scattering Limited spatial resolution (only to micrometers) Extremely small cross section Interference of fluorescence Irreproducible measurement (SERS)	Kumar (2012); Popovic et al. (2011); Chang et al. (2012); Kattumenu et al. (2012); Kneipp et al. (2010); Kumar and Thomas (2011); Mannelli and Marco (2010); Braun et al. (2009); Lin and Chang (2007); Lucas and Riedo (2012); Sinjab et al. (2012); Xiao et al. (2010)
Near-field scanning optical microscopy (NSOM)	Size and shape of nanomaterials	Simultaneous fluorescence and spectroscopy measurement Nano-scaled surface analysis at ambient conditions Assessment of chemical information and interactions at nano-scaled resolution	Long scanning time Small specimen area analyzed Incident light intensity insufficient to excite weak fluorescent molecules Difficulty in imaging soft materials Analysis limited to the nanomaterial surface	Cuche et al. (2009); Kohli and Mittal (2011); Lin et al. (2012); Lucas and Riedo (2012); Pan et al. (2013); Park et al. (2008); Vancso et al. (2005)
Circular dichroism (CD)	Structure and conformational change of biomolecules (*e.g.* protein and DNA) Thermal stability	Nondestructive and prompt technique	Non-specificity of residues involved in conformational change Less sensitive than absorption methods Weak CD signal for non-chiral chromophores Challenging for analysis of molecules containing multiple chiral chromophores	Caminade et al. (2005); Ghosh et al. (2007); Huang et al. (2013b); Jiang et al. (2004); Knoppe et al. (2010); Kobayashi et al. (2011); Liu and Webster (2007); Ranjbar and Gill (2009); Ratnikova et al. (2011); Sapsford et al. (2011); Shang et al. (2007)
Mass spectroscopy (MS)	Molecular weight Composition Structure Surface properties (secondary ion MS)	High accuracy and precision in measurement High sensitivity to detection (a very small amount of sample required)	Expensive equipment Lack of complete databases for identification of molecular species Limited application to date in studying nanomaterial-bioconjugates	Gmoshinski et al. (2013); Knoppe et al. (2010); Lavigne et al. (2013); Sapsford et al. (2011); Tang et al. (2010); Tiede et al. (2008)
Infrared spectroscopy (IR) Attenuated total reflection Fourier transform infrared (ATR–FTIR)	Structure and conformation of bioconjugate Surface properties (ATR–FTIR)	Fast and inexpensive measurement Minimal or no sample preparation requirement (ATR–FTIR) Improving reproducibility (ATR–FTIR) Independence of sample thickness (ATR–FTIR)	Complicated sample preparation (IR) Interference and strong absorbance of H_2O (IR) Relatively low sensitivity in nanoscale analysis	Gun'ko et al. (2009); Johal (2011); Kane et al. (2009); Kazarian and Chan (2006); Liu and Webster (2007); Zak et al. (2011); Zhao et al. (2008)
Scanning electron microscopy (SEM) Environmental SEM (ESEM)	Size and size distribution Shape Aggregation Dispersion	Direct measurement of the size/size distribution and shape of nanomaterials High resolution (down to sub-nanometer) Images of biomolecules in natural state provided using ESEM	Conducting sample or coating conductive materials required Dry samples required Sample analysis in non-physiological conditions (except ESEM) Biased statistics of size distribution in heterogeneous samples Expensive equipment Cryogenic method required for most NP-bioconjugates Reduced resolution in ESEM	Bernier et al. (2012); Boguslavsky et al. (2011); Bootz et al. (2004); Hall et al. (2007); Jin et al. (2010); Johal (2011); Ratner et al. (2004); Sapsford et al. (2011); Tiede et al. (2008)
Transmission electron microscopy (TEM)	Size and size distribution Shape heterogeneity Aggregation Dispersion	Direct measurement of the size/size distribution and shape of nanomaterials with higher spatial resolution than SEM Several analytical methods coupled with TEM for investigation of electronic structure and chemical composition of nanomaterials	Ultrathin samples in required Samples in nonphysiological condition Sample damage/alternation Poor sampling Expensive equipment	Cuche et al. (2009); Domingos et al. (2009); Dominguez-Medina et al. (2012); Hall et al. (2007); Khatun et al. (2012); Pan et al. (2013); Patri et al. (2006); Schacher et al. (2009); Tiede et al. (2008); Wagner et al. (2007); Wang (2001); Williams and Carter (2009)

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Table 1 (*continued*)

Techniques	Physicochemical characteristics analyzed	Strengths	Limitations	Refs
Scanning tunneling microscopy (STM)	Size and size distribution Shape Structure Dispersion Aggregation	Direct measurement High spatial resolution at atomic scale	Conductive surface required Surface electronic structure and surface topography unnecessarily having a simple connection	Fleming et al. (2009); Kocum et al. (2004); Nakaya et al. (2011); Ong et al. (2013); Overgaag et al. (2008); Wang and Chu (2013)
Atomic force microscopy (AFM)	Size and size distribution Shape Structure Sorption Dispersion Aggregation Surface properties (modified AFM)	3D sample surface mapping Sub-nanoscaled topographic resolution Direct measurement of samples in dry, aqueous or ambient environment	Overestimation of lateral dimensions Poor sampling and time consuming Analysis in general limited to the exterior of nanomaterials	Domingos et al. (2009); Gmoshinski et al. (2013); Mavrocordatos et al. (2004); Parot et al. (2007); Sapsford et al. (2011); Schaefer et al. (2012); Tang et al. (2010); Tiede et al. (2008); Yang et al. (2005)
Nuclear magnetic resonance (NMR)	Size (indirect analysis) Structure Composition Purity Conformational change	Non-destructive/non-invasive method Little sample preparation	Low sensitivity Time consuming Relatively large amount of sample required Only certain nuclei NMR active	Lundqvist et al. (2005); Mullen et al. (2010); Pan et al. (2006); Patri et al. (2006); Tomalia et al. (2003); Valentini et al. (2004) Gun'ko et al. (2009); Mirau et al. (2011); Sapsford et al. (2011); Tang et al. (2010)
X-ray diffraction (XRD)	Size, shape and structure for crystalline materials	Well-established technique High spatial resolution at atomic scale	Limited applications in crystalline materials Only single conformation/binding state of sample accessible Low intensity compared to electron diffraction	Caminade et al. (2005); Cao (2004); Gun'ko et al. (2009); Sapsford et al. (2011); Zak et al. (2011); Zanchet et al. (2001); Zhao et al. (2008); Zhou et al. (2012)
Small-angle X-ray scattering (SAXS)	Size/size distribution Shape Structure	Non-destructive method Simplification of sample preparation Amorphous materials and sample in solution accessible	Relatively low resolution	Doniach (2001); Grosso et al. (2011); Hummer et al. (2012); Rao and Biswas (2009); Sapsford et al. (2011)

The presence of pharmaceutical impurities may significantly impact drug efficacy or even introduce unfavorable side effects. In general, determination of nanomaterial purity can be accomplished through analysis of their chemical compositions. Prior to finalizing a nanomaterial's formulation and proceeding with the composition analysis, proper purification processes are required to remove any residual

Table 2
Physicochemical characteristics of nanomaterials and suitable evaluation modalities.

Nanomaterial characteristics	Techniques	Refs
Size/size distribution	DLS, FCS, RS, NSOM, SEM, TEM, STM, AFM, NMR, TOF-MS, XRD, SAXS, FS, UV–visible, AUC, GE, CE, FFF	Biju et al. (2010b); Bootz et al. (2004); Braun et al. (2009); Caminade et al. (2005); Domingos et al. (2009); Hall et al. (2007); Hurst et al. (2006); Jiang et al. (2004); Mavrocordatos et al. (2004); Murdock et al. (2008); Nienhaus et al. (2013); Pan et al. (2013); Powers et al. (2006); Rao and Biswas (2009); Sapsford et al. (2011); Schacher et al. (2009); Valentini et al. (2004); Wang and Chu (2013); Zanchet et al. (2001)
Surface charge	Zeta potential (ELS), ATR–FTIR, GE, CE	Choi et al. (2011); Liu and Webster (2007); Sapsford et al. (2011); Xu (2008)
Shape	NSOM, SEM, TEM, STM, AFM, XRD, SAXS, AUC	Bootz et al. (2004); Caminade et al. (2005); Hall et al. (2007); Mavrocordatos et al. (2004); Rao and Biswas (2009); Sapsford et al. (2011); Wang and Chu (2013); Zanchet et al. (2001)
Structure	TERS, CD, MS, IR, STM, AFM, RS, NMR, XRD, SAXS, FS, DSC, AUC	Bothun (2008); Caminade et al. (2005); Gmoshinski et al. (2013); Grosso et al. (2011); Gun'ko et al. (2009); Mavrocordatos et al. (2004); Mirau et al. (2011); Mullen et al. (2010); Ong et al. (2013); Popovic et al. (2011); Rao and Biswas (2009); Sapsford et al. (2011); Tomalia et al. (2003); Wang and Chu (2013); Zanchet et al. (2001)
Composition	MS, NMR	Gmoshinski et al. (2013); Mullen et al. (2010); Tomalia et al. (2003)
Purity	MS, NMR, HPLC, HDC	Liu et al. (2012); Mullen et al. (2010); Patri et al. (2006); Sapsford et al. (2011); Tang et al. (2010); Tomalia et al. (2003)
Stability	Zeta potential measurement, CD, TGA, DSC, ITC, thermophoresis, HPLC, HDC	Bothun (2008); das Neves et al. (2010); Gugulothu and Patravale (in press); Khatun et al. (2012); Patri et al. (2006); Sapsford et al. (2011)
Dispersion	ESEM, TEM, STM, AFM	Bernier et al. (2012); Bootz et al. (2004); Hall et al. (2007); Mavrocordatos et al. (2004); Sapsford et al. (2011); Wang and Chu (2013)
Surface properties	CD coupled with an enzyme-linked immunosorbent assay, time-of-flight secondary ion MS, ATR–FTIR, modified AFM, X-ray photoelectron spectroscopy	Baer (2012); Fujie et al. (2009); Guay-Bégin et al. (2011); Liu and Webster (2007); Yang and Watts (2005)
Protein corona (thickness and density)[a]	DLS, FCS, TEM, size exclusion chromatography, differential centrifugal sedimentation	(Milani et al. (2012); Nienhaus et al. (2013); Rahman et al. (2013); Röcker et al. (2009); Walczyk et al. (2010)
Protein corona (composition and quantify)[a]	Polyacrylamide GE, LC–MS/MS	(Cedervall et al. ()2007; Kapralov et al. (2012); Milani et al. (2012); Monopoli et al. (2011); Rahman et al. (2013); Sacchetti et al. (2013)
Protein corona (conformation)[a]	CD, simulation	Gebauer et al. (2012); Laera et al. (2011); Rahman et al. (2013)
Protein corona (affinity)[a]	Size exclusion chromatography, SPR, ITC	Casals et al. (2010); Cedervall et al. (2007); Liu et al. (2013); Rahman et al. (2013); Tassa et al. (2009); Zhao et al. (2013)

[a] Courtesy of Rahman et al. (2013).

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manufacturing components or side products to ensure the absence of endotoxin contamination (Crist et al., 2013).

2.5. Stability

Pharmaceutical stability refers to retaining the same properties for a period of time after the pharmaceutical is manufactured. Similar to conventional single-molecule pharmaceuticals, the stability of nanomedicines may be affected by one or more factors, such as temperature, moisture, solvents, pH, particle/molecular size, exposure to different types of ionizing and non-ionizing radiation, enzymatic degradation and even the presence of other excipients and impurities (Briscoe and Hage, 2009; Patri et al., 2006). The stability of nanomaterial may impact its corresponding toxicity; for instance, a number of studies have shown that quantum dot cytotoxicity might be induced during synthesis, storage or even *in vivo* by oxidative or photolytic degradation of quantum dots (Hardman, 2006).

2.6. Interaction between nanomaterials and biological environments

When nanomaterials are introduced into biological environments or integrated in biomaterials, many undesirable effects such as aggregation, coagulation and non-specific absorption can occur. These may be due to a variety of intermolecular interactions occurring at the interfaces of nanomaterials with biomolecules and interaction-mediating fluids (Nel et al., 2009). While the surface properties of nanomaterials in a given medium are characterized by their physicochemical properties, including chemical composition, shape, surface geometry and crystallinity, porosity, heterogeneity and hydrolytic stability, other properties, such as surface charge, dissolution, hydration, size distribution, dispersion stability, agglomeration and aggregation of nanomaterial, are mainly governed by ionic strength, pH, temperature and the presence of biological or organic macromolecules (French et al., 2009; Hull and Bowman, 2009; Nel et al., 2009; Oberdorster et al., 2005). Thus, appropriate physicochemical characterization of nanomaterials should be profiled based on different physical states of the nanomaterials, such as solution, suspension or dry powder, as well as before and after exposure to the *in vitro* or *in vivo* test environment (Hull and Bowman, 2009).

Techniques for determining the shelf life of nanomaterial formulations are essential before considering the manufacture and use of nanomedicines. For example, it is important to guard against degradation of the nanomaterials caused by moisture, oxidation and/or aggregation. In this respect, the different characterization techniques will be useful for quality assurance.

3. Modalities for physicochemical characterization

Characterization of conventional pharmaceuticals and nanomedicines is based on the evaluation of physicochemical properties such as molecular weight, identity, composition, purity, stability and solubility. Many techniques that are routinely applied for characterization of conventional pharmaceuticals can also be used for characterization of nanomedicines (Patri et al., 2006). Yet, several specific characteristics of nanomaterials such as size, surface composition, surface energy, surface charge and shape are critically important and need to be well investigated to better comprehend nanomaterials' behaviors *in vivo*. Addressed below are brief descriptions of modalities used to examine the specific physicochemical properties of nanomaterials, and their main strengths and limitations for nanomaterial investigation.

3.1. Near-field scanning optical microscopy (NSOM)

The far-field imaging resolution of a conventional optical microscope is limited by the diffraction phenomenon of illuminating light, which is specified by the Rayleigh criterion (Hartschuh, 2008; Heinzelmann and Pohl, 1994). While visible light is used in conventional optical microscopes, any two point sources cannot be resolved if they are spatially separated by less than approximately 200 nm (Heinzelmann and Pohl, 1994). Therefore, optical microscopy is not suitable for nanostructure investigation. NSOM is a surface probe microscopy (SPM) technique that comprises concepts from both SPM and optical microscopy to surpass the far-field resolution limit (Durig et al., 1986; Hayazawa et al., 2012). Instead of equipping an objective lens, essential in a conventional microscope, NSOM permits laser light guided in optical fiber to emit through the tip aperture at close proximity to the object (Durig et al., 1986; Hayazawa et al., 2012). While the aperture radius is smaller than the light wavelength, the light emerging from the aperture becomes evanescent in the near-field distance to the object, meaning that light field is highly confined and localized at the aperture or at the object; therefore, the spatial resolution becomes a function of the aperture size, not the diffraction limit (Hayazawa et al., 2012; Heinzelmann and Pohl, 1994).

Given the advantages of an ensemble of fluorescence and spectroscopy measurements, plus high-resolution topographic information on the surface of nanomaterials, NSOM can access not only phase contrast, polarization, fluorescence and staining that are accessible by conventional optical microscopy, but also the distribution of single molecules on the surfaces of cells and interactions in protein–NP conjugates at nano-scaled spatial resolution (Hinterdorfer et al., 2012; Ianoul and Johnston, 2007; Park et al., 2008; Song et al., 2011; Vancso et al., 2005). Some tradeoffs of implementing NSOM include lengthy scanning time for high resolution images or large specimen area, low incident light intensity hindering excitation of weak fluorescent molecules, difficulty in imaging soft materials caused by the high spring constants of the optical fibers, particularly in shear-force mode, and the ability to only image surface features (Kohli and Mittal, 2011).

3.2. Scanning electron microscopy (SEM)

In contrast to optical microscopy, which uses light sources and glass lenses to illuminate specimens to produce magnified images, electron microscopy (EM) uses beams of accelerated electrons and electrostatic or electromagnetic lenses to generate images of much higher resolution, based on the much shorter wavelengths of electrons than visible light photons. SEM is a surface imaging method in which the incident electron beam scans across the sample surface and interacts with the sample to generate signals reflecting the atomic composition and topographic detail of the specimen surface (Hall et al., 2007; Johal, 2011; Ratner et al., 2004). The incident electrons cause emissions of elastic scattering of electrons, referring to backscattered electrons, inelastic scattering of electrons named low-energy secondary electrons, and characteristic X-ray light called cathodoluminescence from the atoms on the sample surface or near-surface material (Johal, 2011). Among these emissions, detection of the secondary electrons is the most common mode in SEM and can achieve resolution smaller than 1 nm (Johal, 2011).

The size, size distribution and shape of nanomaterials can be directly acquired from SEM; however, the process of drying and contrasting samples may cause shrinkage of the specimen and alter the characteristics of the nanomaterials (Bootz et al., 2004; Hall et al., 2007). In addition, while scanned by an electron beam, many biomolecule samples that are nonconductive specimens tend to acquire charge and insufficiently deflect the electron beam, leading to imaging faults or artifacts. Coating an ultrathin layer of electrically conducting material onto the biomolecules is often required for this sample preparation procedure (Hall et al., 2007; Suzuki, 2002). Because a cryogenic freezing method is often required in EM to image surface groups attached to NPs, the size of nanomaterial cannot be investigated in physiological conditions (Hall et al., 2007). An exception is environmental SEM (ESEM), through which samples can be imaged in their natural state without modification or preparation (Sapsford et al., 2011; Tiede et al., 2008). Because the sample chamber of ESEM is operated in a low-pressure gaseous

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environment of 10–50 Torr and high humidity, the charging artifacts can be eliminated, and coating samples with a conductive material is no longer necessary (Tiede et al., 2008). Still, most of the EM techniques, including SEM, possess the disadvantage of a destructive sample preparation, prohibiting its analysis by other modalities (Gmoshinski et al., 2013). In addition, biased statistics of size-distribution of heterogeneous samples is unavoidable in SEM due to the small number of sample particles in the scanning region (Bootz et al., 2004).

3.3. Transmission electron microscopy (TEM)

As the most frequently used technique for characterizing nanomaterials in EM, TEM provides direct images and chemical information of nanomaterials at a spatial resolution down to the level of atomic dimensions (<1 nm) (Patri et al., 2006; Wang, 2001). In the conventional TEM mode, an incident electron beam is transmitted through a very thin foil specimen, during which the incident electrons interacting with specimen are transformed to unscattered electrons, elastically scattered electrons or inelastically scattered electrons (Williams and Carter, 2009). The magnification of TEM is mainly determined by the ratio of the distance between objective lens and the specimen and the distance between objective lens and its image plane (Williams and Carter, 2009). The scattered or unscattered electrons are focused by a series of electromagnetic lenses and then projected on a screen to generate an electron diffraction, amplitude-contrast image, a phase-contrast image or a shadow image of varying darkness according to the density of unscattered electrons (Williams and Carter, 2009).

In addition to the high spatial resolution of TEM that enhances the morphological and structural analyses of nanomaterials, a wide variety of analytical techniques can be coupled with TEM for different applications; for example, chemical analyses of electron energy loss spectroscopy and energy dispersive X-ray spectroscopy can quantitatively investigate the electronic structure and chemical composition of the nanomaterials, respectively (Patri et al., 2006; Tiede et al., 2008; Wang, 2001). Overall, both TEM and SEM can reveal the size and shape heterogeneity of nanomaterials, as well as the degrees of aggregation and dispersion. TEM has advantages over SEM in providing better spatial resolution and capability for additional analytical measurements (Hall et al., 2007). There are certain drawbacks accompanying the advantages of TEM (Williams and Carter, 2009). A significant tradeoff is that a high vacuum and thin sample section are required for electron-beam penetration in TEM measurement (Hall et al., 2007). Sample destruction and measurement in unnatural/non-physiological conditions are common to all EM techniques. In general, high-resolution EM imaging enables examination of a minute part of the specimen over a certain period of time and results in poor statistical sampling. Also, abundant artifacts are generated due to 3D specimens being probed by the 2D TEM technique in transmission view, leading to no depth sensitivity for a single TEM image. Another limitation is that specimens have to be thin enough to transmit sufficient electrons to produce images; in particular cases, the specimen thickness of less than 50 nm is required while doing high-resolution TEM or electron spectroscopy. The extensive preparation of thin specimens increases the possibility of altering sample's structure and makes TEM analysis a very time consuming process. Another big concern is that TEM specimens can be damaged or even destroyed by intense, high-voltage electron beams.

Interestingly, wet TEM can be used for determining the particle size, dispersion, aggregation/agglomeration and dynamic displacement of nanomaterials in an aqueous environment (Carlton and Ferreira, 2012; Chen and Wen, 2012; Hondow et al., 2012). In addition to adapting the function of ESEM for observing samples under partial water vapor pressure in the microscope specimen chamber, a recently developed wet scanning transmission electron microscopy (STEM) imaging system enables transmission observation of species totally submerged in a liquid phase, compared with the issues of poor contrast and possible drifting of objects occurring in the images of the top surface of the liquid using ESEM (Bogner et al., 2005; Ponce et al., 2012). Thus, the wet mode STEM permits observation in nanoscale resolution and high contrast even through several micrometers of water, without adding contrast agents and stains (Bogner et al., 2005; de Jonge and Ross, 2011).

3.4. Scanning tunneling microscopy (STM)

As the earliest developed technique in the SPM family, STM uses quantum tunneling current to generate electron density images for conductive or semiconductive surfaces and biomolecules attached on conductive substrates at the atomic scale (Albrecht et al., 1988; Avouris, 1990; Binnig and Rohrer, 1983; Miles et al., 1990). Adapting the generic principle for all SPM techniques, *i.e.* bringing a susceptible probe in close proximity to the surface of an object measured to monitor the reactions of the probe (Chi and Röthig, 2001), the essential components of an STM include a sharp scanning tip, an xyz-piezo scanner controlling the lateral and vertical movement of the tip, a coarse control unit positioning the tip close to the sample within the tunneling range, a vibration isolation stage and feedback regulation electronics (Wiesendanger, 1994). As the tip–sample separation is maintained in the range of 4–7 Å, a small voltage applied between the scanning tip and the surface causes tunneling of electrons by which variation of the responding current can be recorded while the tip moves across the sample in the x–y plane to generate a map of charge density (Bonnell, 2001). Alternatively, keeping the responding current unchanged by adjusting the tip height through the use of feedback electronics can generate an image of tip topography across the sample (Bonnell, 2001).

As for characterization of biomolecules using STM or EM techniques, the samples are usually embedded into a matrix to preserve their original conformations, followed by coating the samples with a thin metallic layer, such as gold, before acquiring images (Kocum et al., 2004). It is impossible to image these biomolecules in their native conditions using conventional EM techniques that usually accompany a time-consuming sample preparation procedure. STM, on the other hand, can not only diminish the disadvantages of the EM techniques but also provide an image with atomic scale resolution by, for example, using a Pt–Ir tip with a very sharp end (Kocum et al., 2004). Although the high spatial resolution of STM should benefit the characterization of nanoscale biomaterials such as size, shape, structure, and states of dispersion and aggregation, only few studies using gold or carbon as substrates have been reported (Wang and Chu, 2013). The practical obstacles are mainly due to requirements of the conductive surface of the sample and detection of the surface electronic structure (Wang and Chu, 2013). Unfortunately, most biomaterials are insulating, and a simple connection of the sample's surface electronic structure with its surface topography may not necessarily exist. Still, STM is a preferred tool for investigating conductive atomic structures of, for example, carbon nanotubes, fullerenes and graphene (Wang and Chu, 2013).

3.5. Atomic force microscopy (AFM)

Unlike STM, AFM does not require oxide-free, electrically conductive surfaces for measurement and is a SPM imaging tool consisting of a micro-machined cantilever (typically made of silicon or silicon nitride) with a sharp tip at one end to detect the deflection of the cantilever tip caused by electrostatic and van der Waals repulsion, as well as attraction between atoms at the tip and on the measured surface (Gadegaard, 2006; Hansma et al., 1988; Marti et al., 1988; Ratner et al., 2004). The oscillating cantilever then scans over the surface of specimen to generate an image with a vertical resolution of around 0.5 nm (Tiede et al., 2008; Zhu et al., 2011). Like SEM and TEM techniques, AFM can be used for investigating the size, shape, structure, sorption, dispersion and aggregation of nanomaterials — the different scanning modes employed in AFM studies include noncontact mode

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(also called static mode), contact mode and intermittent sample contact mode (also called dynamic mode and tapping mode) (Hinterdorfer et al., 2012; Mavrocordatos et al., 2004; Picas et al., 2012; Sapsford et al., 2011; Song et al., 2011). In addition to probing the sizes and shapes of nanomaterials under physiological conditions, AFM is capable of characterizing dynamics between nanomaterials in biological situations, such as observing the interaction of nanomaterials with supported lipid bilayers in real time, which is not achievable with current EM techniques (Patri et al., 2006).

AFM is gaining importance due to its capability for imaging biomaterials without causing appreciable damage to many types of native surfaces (Parot et al., 2007; Yang et al., 2005). The main strength of AFM is its capability to image a variety of biomaterials at the sub-nanometer scale in aqueous fluids (Parot et al., 2007). However, a major drawback is that the size of the cantilever tip is generally larger than the dimensions of the nanomaterials examined, leading to unfavorable overestimation of the lateral dimensions of the samples (Gmoshinski et al., 2013; Tiede et al., 2008). Unlike fluorescence techniques, AFM lacks the capability of detecting or locating specific molecules; however, this disadvantage has been eliminated by recent progress in single-molecule force spectroscopy with an AFM cantilever tip carrying a ligand, a cell adhesion molecule or chemical groups, which can probe or detect single functional molecules on cell surfaces (Dufrêne and Garcia-Parajo, 2012; Francius et al., 2008).

3.6. Dynamic light scattering (DLS)

Several physicochemical characteristics of nanomaterials including hydrodynamic size, shape, structure, aggregation state, and biomolecular conformation can be explored using radiation scattering techniques (Inagaki et al., 2013; Sapsford et al., 2011). DLS, one of the most popular light scattering modalities, can probe the size distribution of small particles, molecules or polymers at the scale from submicron down to one nanometer in solution or suspension using a monochromatic light source, *e.g.* a laser (Patri et al., 2006; Sapsford et al., 2011). The principle of DLS is to monitor the temporal fluctuation of the elastic scattering intensity of light, *i.e.*, Rayleigh scattering, induced from the Brownian motion of the particles/molecules of a size much smaller than the incident light wavelength, at a fixed scattering angle (Brar and Verma, 2011; Sapsford et al., 2011). The intensity fluctuation trace comprises a mixture of the constructive and destructive interferences of the scattered light, through which the particle size can be derived from analysis of the motion-dependent autocorrelation function using the Stokes–Einstein equation (Brar and Verma, 2011; Pons et al., 2006b; Sapsford et al., 2011).

For physicochemical characterization of nanomaterials, the main strengths of DLS include its noninvasive manner, short experiment duration (in minutes), accuracy in determining the hydrodynamic size of monodisperse samples, and capabilities of measuring diluted samples, analyzing samples in a wide range of concentrations and detecting small amounts of higher molecular weight species, along with lower apparatus costs and more reproducible measurement than other methods (Brar and Verma, 2011; Filipe et al., 2010; Lim et al., 2013). However, the functions of DLS are impacted by several disadvantages, such as difficulty in correlating size fractions with a particular composition when certain amounts of aggregates are present, dust particles interfering in the scattering intensity, and a relatively small range of particle or molecule size (1 nm–3 μm), although the scale limitation is not really a pitfall for characterization of nanomaterials (Bootz et al., 2004; Brar and Verma, 2011; Filipe et al., 2010). In addition, DLS has limited utility for analysis of samples with heterogeneous size distributions, and resolving the dimensions of a mixed sample population varying in size less than a factor of three; moreover, DLS is unsuited to accurately measuring the sizes of non-spherical nanomaterials because spherical nature of particles is already assumed in the analysis (Bootz et al., 2004; Brar and Verma, 2011; Filipe et al., 2010; Uskokovic, 2012).

3.7. Fluorescence correlation spectroscopy (FCS)

Similar in function to DLS, which detects spontaneous intensity fluctuation caused by molecular diffusion, aggregation or interaction with respect to time, FCS can yield quantitative information such as diffusion coefficients, hydrodynamic radii, average concentrations and kinetic chemical reaction rates through autocorrelation analysis of temporal fluorescent variation by fitting an appropriate model (Krichevsky and Bonnet, 2002; Magde et al., 1972; Sapsford et al., 2011; Wu et al., 2008). Most FCS measurements to date are performed in an optimum detection volume defined by a diffraction-limited spot generated by the strongly focused light in confocal microscopy or two-photon excitation microscopy and thus, only few fluorophores within the illuminated region are excited to restrain a small number of molecules and a high amplitude of correlation function (Krichevsky and Bonnet, 2002; Petryayeva et al., 2013; Schwille, 2001).

Analysis of the binding kinetics between donor and receptor, for example, between nanoscale vesicles and peptides and between quantum dots and proteins, can be approached using FCS or its derivatives, such as a dual-color FCS that cross-correlates data from two different fluorescent channels simultaneously (Boukari and Sackett, 2008; Pons et al., 2006a; Rusu et al., 2004; Sapsford et al., 2011). One significant advantage of FCS over DLS or NMR is the requirement of only a small amount of fluorescent probe particles at sub- to nanomolar concentrations, specifically monitoring the probe particles and preventing interfering contribution from the medium, and probing nanomaterials' dimensions in a range of nanometers to hundreds of nanometers (Boukari and Sackett, 2008). However, retaining the advantages of FCS described above requires selection of a fluorophore with high extinction coefficient, high quantum yield, low singlet-to-triplet state transition probability and low photobleaching (Boukari and Sackett, 2008). Moreover, the lack of models also limits the application and accuracy of FCS. A recent development of FCS–NSOM, which can be applied for examining cell membranes, uses the evanescent axial excitation to constrain the fluorescent background from cytoplasm components in order to achieve an observation area in an order of magnitude below the diffraction limit, with a power density comparable to confocal FCS.(Francius et al., 2008; Vobornik et al., 2008).

3.8. Raman scattering (RS)

RS is a widely-used tool for structural characterization of nanomaterials and nanostructures that provides submicron spatial resolution for light-transparent material without the requirement of sample preparation, making it suitable for *in situ* experiments (Popovic et al., 2011). The principle of RS is to measure the inelastic scattering of photons possessing different frequencies from the incident light after interacting with electric dipoles of the molecule (Cantor and Schimmel, 1980). The process of RS results in frequency differences between the incident photons and the inelastically scattered photons associated with the characteristics of the molecular vibrational states, during which the inelastically scattered photons emitting frequencies lower than the incident photons refer to the Stokes lines in Raman spectrum and the inelastically scattered photons emitting frequencies higher than the incident photons are named Anti-Stokes lines (Cantor and Schimmel, 1980). RS is generally considered to be complementary to IR spectroscopy, *i.e.*, vibrational modes that are Raman active should be IR inactive, and vice versa, for small symmetrical molecules, because Raman transitions result from nuclear motion modulating the polarizability of the molecules, rather than a net change in the dipole moment of the molecules (Cantor and Schimmel, 1980).

One of the major advantages of RS is that it is suitable for studying biological samples in aqueous solution because water molecules tend to be weak Raman scatterers. Furthermore, the detailed molecular information offered by RS can be used to investigate conformations and concentrations of tissue constituents, which demonstrates the

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potential of RS for detecting tissue abnormalities (Kumar, 2012). However, while the conventional RS technique provides indirect characterization of nanomaterials, such as average size and size distribution through analysis of the spectral line broadening and shift, it lacks the spatial resolution necessary to delineate different domains for application in nanotechnology (Kattumenu et al., 2012; Popovic et al., 2011). Other downsides of conventional RS include interference of fluorescence and extremely small cross section, demanding intense laser excitation and a large amount of sample materials to provide sufficient RS signals (Chang et al., 2012). In contrast, implementation of surface enhanced Raman scattering (SERS) can strongly enhance RS signals and increase spatial resolution while the measured biomolecules are adhered to the surface of metallic structures, such as commonly used gold or silver NP colloid substrates (Lee et al., 2013a; Lin et al., 2009; Wilson and Willets, 2013). SERS can be used to (i) study surface functionalization of metallic NPs, (ii) monitor the conformational change in proteins conjugated to the metallic NPs, and (iii) track intracellular drug release from the nanoplatform and measurement of the pH in the surrounding medium (Ando et al., 2013; Huang et al., 2013a; Kneipp et al., 2010; Kumar and Thomas, 2011; Mannelli and Marco, 2010).

By adapting the concept of confining the light field in Raman near-field scanning optical microscopy to overcome diffraction-limited resolution, a recently emerging technique, tip-enhanced Raman spectroscopy (TERS), utilizes an apertureless metallic tip instead of an optical fiber to gain the surface enhancement of the Raman signals (the SERS effect) (Ando et al., 2013; Hartschuh, 2008; Hayazawa et al., 2012; Wang and Irudayaraj, 2013). In contrast to conventional RS, SERS and TERS provide topological information of the nanomaterials, in addition to their structural, chemical and electronic properties, which conventional RS provides (Lee et al., 2013b; Popovic et al., 2011). However, the lack of measurement reproducibility in SERS caused by the size and shape variation, as well as undesirable aggregation of NPs is an obstacle for *in vitro* or *in vivo* imaging applications (Xiao et al., 2010).

3.9. Circular dichroism (CD)

Given a chiral molecule that possesses molecular asymmetry, CD is used to characterize the structure of the molecule through the different absorptions of circularly polarized lights in left-handed direction and in right-handed direction on the asymmetric molecule (Ranjbar and Gill, 2009). In the past few decades, various types of CD-based techniques have been developed to improve the capability of assessing conformational changes in proteins and nucleic acids, secondary and tertiary structures of proteins and their thermal stability, and donor–acceptor interactions, *e.g.* protein–protein, protein–DNA, protein–ligand and DNA–ligand interactions (Jiang et al., 2004; Ranjbar and Gill, 2009; Sapsford et al., 2011; Shang et al., 2007). In addition, the conformational behavior of biomolecules on NPs, the structures of drug-delivery nanocarriers and the interactions of nanocarriers with biomolecules have been investigated using CD techniques (Bhogale et al., 2013; Caminade et al., 2005; Ghosh et al., 2007; Liu and Webster, 2007; Ranjbar and Gill, 2009).

Although conventional CD spectroscopy is a prompt, nondestructive tool to reveal the structure and/or conformational change of the biomolecule investigated, there are several limitations of this technique. First, CD cannot manifest the actual contribution made by any particular amino-acid residue in a protein-type biomolecule to composing a CD spectrum (Ranjbar and Gill, 2009). Second, CD spectroscopy, based on differential absorption of left and right circularly polarized radiation, is less sensitive than absorption spectroscopy by two to three orders of magnitude. Third, it is challenging to analyze CD spectra acquired in a complex of a chiral compartment adhering to a chiral receptor, which is very common in biomacromolecules and nanomaterials. And finally, conventional CD measurement exhibits weak spectra if the sample contains only non-chiral chromophores. Some of the limitations can be eliminated by implementing different CD-based techniques, for example, fluorescence detected CD to enhance sensitivity, and magnetic CD to detect molecules that lack a chiral center (Kobayashi et al., 2011; Tanaka et al., 2005).

A number of CD-based techniques have been developed to improve biological structure measurements, such as electronic CD, magnetic CD (MCD), fluorescence detected CD, near-infrared CD, vibrational CD (VCD), HPLC–CD, stopped-flow CD and synchrotron radiation CD (Ranjbar and Gill, 2009). Some of these CD-based methods have been used to investigate nanomaterials in various circumstances/situations (Burgi, 2011). For example, the local characteristics of VCD spectra revealed the conformation of 1,1′-binaphthyl-2,2′-dithiol adhered to gold nanoclusters (Gautier and Bürgi, 2010). Additionally, MCD spectroscopy, which is complementary to UV–vis spectroscopy, for the gold(I) complex $Au(AuPPh_3)_8^{3+}$ in a solution phase yielded higher resolution and more features, compared with that of electronic absorption (Yao et al., 2012).

3.10. Infrared (IR) spectroscopy

Typically, a molecule may absorb IR radiation if it possesses a time-variant dipole moment and its oscillating frequency is the same as the frequency of incident IR light (Johal, 2011). The absorption of IR radiation transfers energy to the molecule, inducing a corresponding covalent bond stretching, bending or twisting, which, in the case of a normal mode, is described by a stationary state of molecular vibrational Hamiltonian (Cantor and Schimmel, 1980). Molecules without dipole moments, *e.g.* diatomic molecules N_2 and O_2, do not absorb IR radiation (Johal, 2011). Generally in a molecule, the vibrations involve various coupled pairs of atoms or covalent bonds, each of which must be considered as a combination of the normal modes; therefore, the IR spectrum, illustrating absorption or transmission versus incident IR frequency, can offer a fingerprint of the structure of the molecule of interest (Cantor and Schimmel, 1980).

For nanomaterial applications, Fourier transform infrared (FTIR) spectroscopy is commonly employed to use the expression of characteristic spectral bands to reveal nanomaterial–biomolecule conjugation, *e.g.* proteins bound to NP surfaces, and to illustrate the conformational states of the bound proteins (Jiang et al., 2004; Perevedentseva et al., 2010; Shang et al., 2007; Tom et al., 2006). Furthermore, FTIR has also been extended to study nano-scaled materials, such as confirmation of functional molecules covalently grafted onto carbon nanotubes (Baudot et al., 2010). A recently developed technique called attenuated total reflection (ATR)–FTIR spectroscopy uses the property of total internal reflection in conjunction with IR spectroscopy to probe the structure of adsorbed/deposited species at a solid/air or solid/liquid interface, while avoiding the drawbacks of sample preparation complexity and spectral irreproducibility in conventional IR (Hind et al., 2001; Johal, 2011). In an ATR–FTIR system, the total internal reflectance, occurring within the equipped internal reflection element (IRE) crystal, which has a high refractive index at certain angles, forms evanescent waves that extend from the IRE crystal–sample interface into the sample with penetration depth of micrometers (0.5–5 μm), and the intensity of the evanescent waves decays exponentially from the interface (Johal, 2011). ATR–FTIR can provide IR absorption spectra to investigate, for example, changes in surface properties as well as identification of chemical properties on the polymer surface when sample on the IRE–sample interface absorbs the evanescent IR waves with frequencies matching the vibrational modes of the sample (Johal, 2011; Kazarian and Chan, 2006; Liu and Webster, 2007). Although ATR–FTIR spectroscopy can be implemented to study the surface features of nanomaterials, it is not a very sensitive surface-analysis method at nanometer scale because the penetration depth of ATR–FTIR has the same order of magnitude as the incident IR wavelength (Liu and Webster, 2007).

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3.11. Nuclear magnetic resonance (NMR)

In contrast to imaging and diffraction techniques affording structural information at long-range order, *i.e.* the crystalline property, NMR is sensitive to the local environment to resolve the structures of amorphous materials, polymers and biomolecules that lack long-range order (Wang et al., 2001). In addition to evaluating the structures and compositions of the species, NMR spectroscopy provides tools to investigate dynamic interactions of the species in different conditions (Sapsford et al., 2011; Tiede et al., 2008) — the relaxation, molecular conformation and molecular mobility can be evaluated through different dynamic measurements using specifically designed rf and/or gradient pulse sequences (Wang et al., 2001). NMR spectroscopy has been implemented to determine several physiochemical characteristics of nanomaterials, including structure, purity and functionality in dendrimers, polymers and fullerene derivatives, as well as conformational changes occurring in the interactions between ligands and nanomaterials (Lundqvist et al., 2005; Mullen et al., 2010; Pan et al., 2006; Patri et al., 2006; Tomalia et al., 2003). Pulsed field gradient NMR has been implemented to evaluate the diffusivity of nanomaterials, through which the sizes and interactions of species under investigation can be calculated (Valentini et al., 2004).

NMR is a non-destructive/noninvasive technique that requires little sample preparation. However, the low detection sensitivity of NMR, in contrast to optical techniques, requires a relatively large amount of the sample for measurement (Sapsford et al., 2011). It can also be time consuming if a certain level of signal-to-noise ratio is necessary for spectral analysis.

Over the past few years, the method using magic angle spinning for non-solid materials named high-resolution magic angle spinning (HR-MAS) NMR has been widely adapted in the biological and biomedical fields due to its capability of generating spectra similar to high-resolution NMR for investigating tissues and cells with heterogeneous nature (Alam and Jenkins, 2012). The advantage of HR-MAS NMR for accurate characterization of the surface-attached ligands and modified surfaces has been utilized for investigating each synthetic step of the cyclo-peptide immobilized on the surface of poly(vinylidene fluoride) based NPs, and studying thermolytically produced thiol-derivatized silver clusters (Alam and Jenkins, 2012; Conte et al., 2007; Deshayes et al., 2010).

3.12. Mass spectrometry (MS)

MS is one of the major analytical techniques used to examine the mass, elemental composition and chemical structure of a particle or a molecule. The basic principle of MS is to distinguish charged particles with different masses based on their mass-to-charge ratios (McNaught and Wilkinson, 1997). MS provides a high degree of precision and accuracy for molecular weight determination, as well as high detection sensitivity, which only requires 10^{-9} to 10^{-21} mol of a sample. Several physicochemical characteristics of nanomaterials, including mass, composition and structure, can be depicted using various MS procedures, distinguished by their ion sources, separation methods and detector systems (Gmoshinski et al., 2013). Among the ionization techniques coupled with MS analyzers, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are commonly used to ionize and volatilize the thermally-labile biomolecular derivatives instead of introducing significant fragmentation or decomposition of the molecules. Inductively coupled plasma (ICP) ionization, on the other hand, is mainly implemented in the analysis of metal-containing nanomaterials (Gmoshinski et al., 2013; Tiede et al., 2008). Applications of different MS procedures for nanomaterials include using time of flight (TOF)-MS to determine the size/size distribution of nanomaterials (Powers et al., 2006), MALDI–TOF-MS to measure the molecular weights of macromolecules, polymers and dendrimers as well as to illustrate proteins binding to NPs (Patri et al., 2006; Tom et al., 2006), ICP-MS to validate the conjugation reaction of a functionalized NP with a modified contrast agent (Endres et al., 2007), and secondary ion MS to access the elemental and molecular properties of the top layer of NPs, as well as to examine biomaterial surface properties in physiological conditions (Guo et al., 2006; Ratner et al., 2004). Although these MS techniques have been applied to the analysis of physicochemical properties of various biomolecules, the currently incomplete MS spectral databases still cause difficulty in identifying molecular species, for example, in the analysis of MALDI–TOF-MS outcome measures (Lavigne et al., 2013). Additionally, the applications of MS techniques for nanomaterials to date are constrained in nanomaterial-bioconjugate characterization, mainly due to the cost of instrumentation, sample destruction and necessary instruments generally supplied for other investigations (Sapsford et al., 2011).

3.13. Zeta potential

In an ionic solution, the surface of a charged particle is firmly bound to opposite charged ions, forming a thin liquid layer named the Stern layer, which is encompassed by an outer diffuse layer consisting of loosely associated ions. These two layers compose the so-called electrical double layer (Clogston and Patri, 2011). Given the tangential motion driven by an external force or Brownian motion of the charged particle, the movement of the charged particle shears ions migrating with the charge particle in the diffuse layer from ions staying with the bulk dispersant outside the layer (Clogston and Patri, 2011). The electric potential on the shear surface is called zeta potential, which is usually determined by measuring the velocity of the charged species towards the electrode in the presence of an external electric field across the sample solution (Pons et al., 2006b; Sapsford et al., 2011). The zeta potential with a value of ± 30 mV is generally chosen to infer particle stability, through which the absolute value greater than 30 mV indicates a stable condition, whereas a low zeta potential value of less than 30 mV indicates a condition towards instability, aggregation, coagulation or flocculation (Sapsford et al., 2011).

Among the methods of evaluating zeta potential, the technique of electrophoretic light scattering (ELS), which can simultaneously measure the velocities of many charged particles in liquid, is most commonly used (Doane et al., 2011; Xu, 2008). However, it still suffers the electro-osmotic effect that reduces precision and reproducibility of the measurement (Weiner et al., 1993). Although measuring the zeta potential of suspended particles after dilution reduces difficulty of light penetration into the sample solution, it is worth noting that zeta potential is a property sensitive to environmental changes including pH and ionic strength (Weiner et al., 1993; Xu, 2008). Therefore, a precise, repeatable zeta potential measurement in a diluted solution cannot reflect the true value in a concentrated suspension (Xu, 2008).

3.14. X-ray diffraction (XRD)

In a variety of X-ray spectroscopic modalities, XRD is a primary tool for completely resolving the tertiary structures of crystalline materials at the atomic scale (Cantor and Schimmel, 1980; Sapsford et al., 2011). The diffraction of X-ray can be simply described as the reflection of a collimated beam of X-rays incident on the crystalline planes of an examined specimen according to Bragg's law (Cantor and Schimmel, 1980). Typically, XRD, based on wide-angle elastic scattering of X-rays, is a tool for characterizing crystalline size, shape and lattice distortion by long-range order, but is limited to disordered materials (Caminade et al., 2005; Sapsford et al., 2011; Zanchet et al., 2001).

Although XRD is a well-established technique and has frequently been used to determine the material structure at the atomic scale, difficulty in growing crystals and the ability of getting results only from single conformation/binding state of the sample limit the applications of XRD technique (Cao, 2004; Sapsford et al., 2011; Zanchet et al., 2001). Another disadvantage of XRD is the low intensity of diffracted

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X-rays, particularly for low atomic number materials, compared with electron diffractions (Cao, 2004). A recent X-ray diffraction study reported a new approach using femtosecond pulses from a hard-X-ray free-electron laser for structure determination, which may benefit structure determination of macromolecules that do not yield sufficient crystal size for using conventional radiation sources or are not sensitive to radiation damage (Chapman et al., 2011).

3.15. Small-angle X-ray scattering (SAXS)

In contrast to XRD, whose applications are limited to crystalline materials, SAXS provides information of several characteristics by examining either crystalline or amorphous materials from polymers, proteins to nanomaterials (Lipfert and Doniach, 2007; Rao and Biswas, 2009; Sapsford et al., 2011). In SAXS, a portion of an incident X-ray beam elastically scattered from the sample forms a scattering pattern on a two-dimensional flat X-ray detector perpendicular to the direction of the incident X-ray beam (Doniach, 2001; Rao and Biswas, 2009; Sapsford et al., 2011). By analyzing the intensity of the scattered X-ray collected within the scattering angle, ranging from 0.1 to 3°, SAXS can evaluate the size/size distribution, shape, orientation, and structure of a variety of polymers and nanomaterial-bioconjugate systems in solution (Doniach, 2001; Rao and Biswas, 2009; Sapsford et al., 2011).

The features of small-angle scattering in SAXS lead to the capability of studying non-repeating structures; therefore, perfect crystallized structures are not required, which simplifies sample preparation and makes SAXS a non-destructive method (Rao and Biswas, 2009). On the other hand, SAX measurements provide holistic information about the structure, which exhibits the averaged characteristics rather than local probes of individual grains (Rao and Biswas, 2009). This feature can be a disadvantage if high resolution is required. On the other hand, recent progress in SAXS can achieve higher resolution measurements by introducing synchrotron as the high-energy X-ray source (Petoukhov and Svergun, 2013; Rao and Biswas, 2009).

Other X-ray spectroscopic techniques, such as X-ray absorption spectroscopy, can yield information about chemical state and symmetries of the absorption site through analysis of the X-ray absorption near edge structure spectra, and provide structural information, including coordination numbers and inter-atomic distance to ligands and neighboring atoms from the absorbing element through investigation of the spectra of extended X-ray absorption fine structure (EXAFS) without the requirement of long-range order in the measured species (Koningsberger and Prins, 1988; Zanchet et al., 2001). Both XRD and EXAFS can provide the averaged structural information of a nanomaterial, resulting from a long-range order and a local order of samples examined, in the manner of elastic and inelastic X-ray interaction with the samples, respectively (Zanchet et al., 2001).

4. Other techniques

Many other commonly used spectroscopic techniques for investigating the physicochemical characteristics of nanomaterials have not been listed above. One such sample is the use of UV–visible absorbance spectroscopy to investigate the characteristics of nanomaterials including size, concentration, aggregation state and even bioconjugation when the absorption profiles of nanomaterials are distinct (Biju et al., 2010b; Jiang et al., 2004; Sapsford et al., 2011). Fluorescence spectroscopy (FS), in general, is a more effective technique for pursuing the ligand binding or conformational changes of macromolecules than CD and light absorption techniques due to its sensitivity to the environment of the chromophore, as a consequence of the targeted molecular electron remaining in the excited singlet state for a relatively long duration before de-excitation (Cantor and Schimmel, 1980). Furthermore, conjugation of an extrinsic fluorophore to the non-intrinsically fluorescent nanomaterials enables FS to determine the characteristics of biomolecule on the NP surface, including concentration, particle size, and spacer composition (Hurst et al., 2006).

The thermal stability and the amount of the nanomaterial conjugates can be evaluated using several thermal techniques (Sapsford et al., 2011). The temperature-dependent weight change in bulk samples, such as various nanomaterial bioconjugates, can be monitored using thermal gravimetric analysis (TGA) (Gibson et al., 2007; Vaiyapuri et al., 2012). Material transitions such as melting, crystallization, glass transition and decomposition of nanomaterial-bioconjugates can be accessed through differential scanning calorimetry (DSC); therefore, subsequent analysis of DSC measurements can provide the structure and stability of the investigated material (Bothun, 2008). In addition, the stoichiometry, affinity and enthalpy derived from the interaction between nanomaterial and biomolecule can be determined using isothermal titration calorimetry (ITC) (Cedervall et al., 2007). By locally heating the sample to generate a temperature gradient, thermophoresis monitors the motion of the sample to evaluate its size and surface potential (Sapsford et al., 2011; Sperling et al., 2007). However, thermophoresis needs higher concentrations of the examined species than FCS does to ensure robust signals.

Several separation techniques are routinely used as characterization tools. Centrifugation, of course, is a conventional methodology of separating and purifying mixed materials. In the category of centrifugation techniques, analytical ultracentrifugation (AUC) can be implemented to investigate the conformation, structure, stoichiometry and self-aggregation state of nanomaterials, in addition to determining the size/size distribution, shape and molecular weight (Inagaki et al., 2013; Sapsford et al., 2011; Schaefer et al., 2012). While coupled with reverse-phase, ion-exchange-phase or size-exclusion-phase columns, the chromatography techniques, such as high-performance liquid chromatography (HPLC) and hydrodynamic chromatography (HDC), can be used for the purification of nanomaterial bioconjugates. Owning the capability of differentiating different nanomaterial bioconjugates, these chromatography techniques can exhibit the distribution of nanomaterial-to-biomolecule ratios, as well as the stability and purity of the post-products (Patri et al., 2006; Sapsford et al., 2011). Methods of electrophoresis are routinely used to partition and purify biomolecules, and gel electrophoresis (GE) and capillary electrophoresis (CE), for example, can further provide the relative and absolute hydrodynamic size and zeta potential of nanomaterials (Sapsford et al., 2011). Field flow fractionation (FFF), which utilizes an external field such as flow, thermal, electrical and magnetic fields applied to a fluid suspension or solution to separate the particles present in the fluid, has been implemented to reveal the size/size distribution and charge information of the investigated nanomaterials (Sapsford et al., 2011). Sedimentation and flow FFF can exhibit the effective mass, hydrodynamic size, density and volume of the nanomaterials investigated.

5. Characterization of nanomaterials

Nanomaterials commonly consist of at least two of the following units: metallic, semiconducting and organic particles or molecules (Kim et al., 2010). Additionally, nanomaterials are generally coated with polymers or biorecognition molecules to improve biocompatibility and selective targeting of biologic molecules (Kim et al., 2010). A common feature of all nanomaterials is their large ratio of surface area to volume, which may be orders of magnitude greater than that of macroscopic materials. Still, the final size and structure of nanomaterials depend on the salt and surfactant additives, reactant concentrations, reaction temperatures, and solvent conditions used during their synthesis. Stated thus, comprehension of these physicochemical properties as well as the fundamentals of the associated measuring methods is necessary before characterizing nanomaterials and developing reproducible synthesis procedures to optimize the medical application of nanomaterials.

Some nanomaterials that are nanomedicines or considered to be potential nanomedicines are generally split into several categories based

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on the types of nanomaterials or the application areas, such as drug delivery, drugs and therapies, *in vivo* imaging, *in vitro* diagnostics, biomaterials and implants (Wagner et al., 2008). Regardless of what criterion is used to categorize these nanomaterials, they share a certain degree of commonality in their physicochemical characteristics within and across the categories, and the same characteristics in different nanomaterials can be visualized through the use of the same or equivalent techniques described above.

Nano-drug delivery systems aim to optimize bioavailability at particular locations over a period of time, minimizing drug toxicity, increasing drug-therapeutic index and replacing invasive administration routes with non-invasive ones (Goldberg et al., 2007; Wagner et al., 2008). Nano-drug delivery systems include liposomes, nanosuspensions, NPs, dendrimers, fullerenes, and carbon nanotubes and the drug carriers in nano-drug delivery systems can be devised by regulating the composition, size, shape and morphology (Goldberg et al., 2007; Wagner et al., 2008). In a nano-drug delivery system, the system size can influence bioavailability and circulation time in blood stream, partly resulting from the impact of surface area-to-volume ratios on the solubility of the drug delivery systems (Goldberg et al., 2007; Rabinow, 2004; Vinogradov et al., 2002). Studies showed that 10–100 nm is an optimal size for nano-drug delivery systems to mostly avoid rapid removal through extravasation or through phagocytosis (Stolnik et al., 1995; Vinogradov et al., 2002). Recent studies have demonstrated that the shape of the drug carrier plays an important role in biodistribution and cellular uptake as well as avoiding phagocytosis and prolonging circulation in blood stream (Champion and Mitragotri, 2009; Geng et al., 2007). In addition, it has been reported that the surface charge of a nano-drug delivery system affects the pharmacokinetics of drugs entrapped/adhered (Hathout et al., 2007; Law et al., 2000), while the structural difference of the delivery systems may influence drug delivery efficiency (Inokuchi et al., 2010). Among the techniques described in this article for physicochemical characterization, DLS, FCS, RS, NSOM, SEM, TEM, STM, AFM, NMR, XRD, SAXS, FS and several separation techniques are suitable for evaluating the size and size distribution of nano-drug delivery systems. NSOM, SEM, TEM, STM, AFM, XRD and SAXS are proper modalities for shape measurement, while appropriate methods for surface charge measurement include zeta potential measurement (ELS), ATR–FTIR, GE and CE. In addition, TERS, CD, MS, IR, STM, AFM, NMR, XRD, SAXS, FS and some of the thermal and separation techniques can investigate the structural properties of the nanomaterials.

Along with the development of nano-drug carriers, certain types of nanomaterials have been used to design active pharmaceuticals, such as a dendrimer-derived microbiocide for preventing HIV infections and fullerenes for binding and scavenging or inactivating free radicals, which are associated with the induction of neural and cardiovascular diseases (Wagner et al., 2008). Super-paramagnetic iron-oxide NPs coated with aminosilane, for example, can be used in hyperthermia treatment of cancer by subjecting the tumor tissue to high temperatures in order to destroy neoplastic cells (Wagner et al., 2008). Magnetic NPs bound to antibodies can be specific to certain targets, *e.g.*, stem cells, and allow sorting via magnetic field for cell therapy (Wagner et al., 2008). In addition to the physicochemical properties, including size, shape, surface charge and structure mentioned already, the stability, particularly thermal stability, of the nanomaterials plays a crucial role if nano-drugs and nano-formulations are to retain and exert consistent therapeutic efficacy. In this article, the modalities capable of characterizing the stability of nanomaterials are zeta potential measurement, CD, HPLC, HDC and several thermal techniques including TGA, DSC, ITC and thermophoresis.

Molecular diagnostics is aimed at diagnosing disease at a molecular level before symptoms manifest (Wagner et al., 2008). Compared with conventional molecular imaging agents, employment of nanomaterial-based contrast agents generally increases the signal intensity of a single particle (Rosenblum et al., 2010; Thomas et al., 2013). The strong signal generated by the nanomaterial-based contrast agents, in fact, helps overcome the essential disadvantages of low sensitivity in MRI and limited depth penetration of optical imaging to a certain degree (Lam et al., 2013; Rosenblum et al., 2010; Thomas et al., 2013). Given the novel properties of nanomaterials, several distinct nanomaterials are commonly designed as nanoscale imaging probes, including quantum dots with specific electronic and optical properties, upconversion phosphors consisting of phosphor nanocrystals doped with rare earth metals, and super-paramagnetic iron oxide particles containing an iron oxide core of magnetite and/or maghemite encased in polysaccharide, synthetic polymer or monomer coatings, or other soft materials like dendrimers (Biju et al., 2010a; Liang et al., 2008; Rosenblum et al., 2010; Wang et al., 2011). In addition to the characteristics of conventional imaging probes, such as structure, purity and solubility, certain physicochemical properties of nanomaterial-based imaging contrast agents also have to be considered, including size, shape, composition, zeta potential and dispersion (Leung et al., 2012). Techniques that can characterize the property of purity include NMR, HPLC and HDC, while the property of composition can be characterized by MS and NMR. Furthermore, the EM- and SPM-derived techniques, such as ESEM, TEM, STM and AFM, can be implemented to characterize the dispersion of nano-based imaging probes.

Even *in vivo* nanomaterial-based imaging contrast agents are continuously under development, nanomaterial toxicity in the body has not been comprehensively studied (Chi et al., 2012). While toxicity of being a minor concern leads to various types of nanomaterials widely used in the context of *in vitro* diagnostics (Chi et al., 2012), the applications of *in vitro* diagnostics have attracted a large amount of research interests, mainly split into NP-based biomarkers and novel sensor platforms composed of nanomaterials (Chi et al., 2012; Wagner et al., 2008). Among the physicochemical characteristics, stability is a key property in the applications of biomarkers. An example is the complete replacement of organic dyes with inorganic fluorescent NPs because organic dyes in polymerase chain reaction assays and in biochips are not photostable (Chi et al., 2012; Wagner et al., 2008). While biochips with a nano-based electrical detection system are the most popular development in the field of nano-sensor platforms, the surface chemistry properties play an important role in determining the capabilities of the biochips (Chi et al., 2012; Wagner et al., 2008).

Compared to drug delivery studies, the developments of nanoscale biomaterials and implants are still in their infancy. Still, nanomaterials have been used in a wide spectrum of applications, including tissue regeneration and medical implants (Liu and Webster, 2007; Wagner et al., 2008). Nanomaterials have been considered for a variety of implant applications, such as bone substitute materials, cartilage regeneration, vascular graft endothelialization, bladder replacement, dental restoratives, neural prostheses and antibiotic materials (Liu and Webster, 2007; Wagner et al., 2008). Among the physicochemical characteristics, surface properties are of the greatest importance to understand protein-mediated cell responses since the unique surface properties of the nanomaterials can influence interactions with proteins attached to selected cell membrane receptors (Liu and Webster, 2007). Techniques that can provide surface chemical characterization and investigation of protein–nanomaterial interactions include CD coupled with an enzyme-linked immunosorbent assay, time-of-flight secondary ion MS, ATR–FTIR, modified AFM and X-ray photoelectron spectroscopy (Liu and Webster, 2007).

Protein corona is formed in a dynamic, competitive process during which proteins or enzymes present in the biological fluid adhere to the surface of nanomaterials to generate a bio-nano interface (Luyts et al., 2013; Mahmoudi et al., 2011; Nel et al., 2009). The physicochemical properties of nanomaterials influenced by protein corona include surface properties, aggregation properties and hydrodynamic size charge; in the meantime, the adhered proteins can endure conformational alternation, functionality changes, unmasking of new epitopes and alternations in avidity and affinity effects (Cedervall et al., 2007; Luyts et al., 2013; Nel et al., 2009). In contrast to using centrifugation,

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a conventional method that likely disturbs protein–NP complexes, a number of methodologies, including size-exclusion chromatography gel filtration, ITC, surface plasmon resonance (SPR), have been proposed for measuring dynamic and equilibrium parameters of the protein–NP interactions and estimating the potential of NP-associated risks (Cedervall et al., 2007; Dahlin et al., 2013).

6. Conclusion

Given the novelty of physicochemical characteristics at the nanometer scale, nanomaterials have potential to impact physiological interactions from the molecular level to the systemic level, making the *in vivo* administration of nanomedicines an interesting research topic. The rapid development and production of nanomaterials for use as nanomedicines indicate the demand and wisdom for regulating the manufacture and use of nanomaterials. Robust techniques for characterization of nanomaterials are fundamental to regulatory guidelines for ensuring safety of nanomaterials in general and nanomedicines in particular. This article describes the essential physicochemical properties of nanomaterials, followed by an introduction to different methods that are commonly used for characterizing nanomaterials. Indeed, it is necessary to characterize the nanomaterial intended for therapeutic use in both its originally manufactured condition and after introduction into a physiological environment. The brief description of each technique, together with its strengths and limitations, provides us with a picture for selecting suitable techniques for characterization of a potential nanomedicine.

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