Chapter 7. Characterization of Bio-nanosystems

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Abstract

The European Commission and European agencies since 2011 have issued regulatory guidelines and requirements that set the objectives and agenda for the development and implementation of techniques for characterization of nanomaterials in general and of bio-nanomaterials in particular. The physicochemical properties of nanoparticles largely determine their usefulness in applications as well as any potential environmental and health hazards associated with those applications. Accordingly, accurate and reliable nanocharacterization techniques are required to establish, maintain, and control the use of nanomaterials that promotes both commercial and societal benefits. To determine size, size distribution, concentration, composition, and surface properties of nanoparticles, results from multiple complementary techniques need to be combined and jointly interpreted, including those from appropriately adapted variants of microscopy, spectroscopy, spectrometry, diffraction, scattering, absorbance, centrifugation, sedimentation, separation, chromatography, and diffusion measurements. The current state-of-the-art capabilities for such measurements are reviewed in this chapter.

Keywords

nanoparticles, nanocharacterization, size, concentration, composition, surface

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7.1 Introduction

In the context of food research and applications, the objectives of characterizing nanoparticles are primarily derived from the guidance (EFSA et al. 2018) issued by the European Food Safety Authority (EFSA). And while the ultimate properties of interest for food materials and substances are related to their interactions with human senses and bodies, the characterization of these materials—and any nanomaterial components thereof—must begin with their physicochemical properties (Gioria et al. 2018; Rasmussen et al. 2018). The resulting need to adapt and extend various analytical methods from their traditional use on relatively well-defined nanomaterials encountered in physics and chemistry to characterization of the more complex and heterogeneous nanomaterials in food creates well-recognized, if not yet fully understood, challenges (Rasmussen et al. 2018). A provocative editorial recently highlighted this perspective of challenges that arise when working with "messy systems" and trying to obtain reliable and reproducible results (Harris 2017).

These daunting challenges notwithstanding, the regulatory needs (EFSA 2018) and requirements in both food (EFSA et al. 2018) and biomedical (Gioria et al. 2018; Pita et al. 2016) applications of nanomaterials have motivated the development and validation of analytical techniques and methodologies that enable characterization of increasingly more complex nanomaterials (Table 7.1). The most significant progress has been made in characterization of solid nanoparticles that are subject to the more extensive regulations across various application areas. The resulting methodologies are being successfully adapted to related nanomaterials that are composed of a solid core and a shell of "softer" organic or biological material. Important advances are being made in characterization of soft nanoparticles as well, however, both regulators and practitioners agree that the inherent flexibility and responsiveness of soft nanomaterials make them particularly challenging targets for robust and unambiguous measurements.

The two types of nanomaterials outlined above—solid-core and soft nanoparticles—are discussed in the two sections of the following brief overview, with each section organized by the individual physicochemical properties and parameters that need to be characterized within the context of the EFSA guidance (EFSA et al. 2018). More extensive overviews of the physicochemical properties and characterization methods have been recently provided for edible

nanoparticles (McClements and McClements 2013) and for general nanocharacterization, based on the Organisation for Economic Co-operation and Development (OECD) Testing Program (Rasmussen et al. 2018).

Table 7.1 International projects addressing regulatory and metrology aspects of nanocharacterization

Name	URL	Description
NANoREG	www.nanoreg.eu	A common European approach to the regulatory testing of manufactured nanomaterials.[a]
DaNa$^{2.0}$	www.nanopartikel.info/en/	Information about nanomaterials and their safety assessment.
NanoDefine	www.nanodefine.eu	Methods for the implementation of the European definition of a nanomaterial.[b]
InNanoPart	empir.npl.co.uk/innanopart	Measuring the concentration and surface chemistry of nanoparticles.

[a]The Dutch National Institute for Public Health and the Environment (RIVM) maintains the *NANoREG Results Repository*: http://www.rivm.nl/en/About_RIVM/International/International_Projects/Completed/NANoREG

[b]Results from the NanoDefine project are summarized in Babick et al. 2016 and Wohlleben et al. 2017.

7.2 Solid-core Nanoparticles

Among various complex nanomaterials, solid-core nanoparticles benefit from availability and applicability of some of the most advanced nanocharacterization techniques, many of which have been adapted from the decades of extensive research on characterization of solid nanoparticles (Bowen 2002). Solid nanoparticles are typically sufficiently stable in terms of size, morphology, and composition that the appropriate variants of electron microscopy, mass spectrometry, spectroscopy, adsorption, diffraction, light scattering, gravimetry, thermal, and sedimentation measurements can be used to obtain complementary information about their properties (Rasmussen et al. 2018). And while only some of the methods, typically those that are optimized for speed and simplicity rather than high information content, can be readily employed by practitioners in research and industry, the more advanced and information-rich techniques are available as an analytical "backstop," when lower uncertainty is required or ambiguity/inconsistency in results needs to be resolved. Serendipitously, the insight into the

properties of a nanomaterial derived from long-term multi-technique characterization (Nurmi et al. 2005) often can be used to identify the two or three techniques that provide sufficient information for routine analysis of the material (Baer et al. 2013).

In characterization of solid-core nanoparticles, the explicit inclusion of surface modification and coatings into consideration requires the use of surface-analysis techniques (Baer and Engelhard 2010; Baer et al. 2010; Rasmussen et al. 2018) and the consideration of how the changes in the environment and preparation of nanoparticles affect their surfaces (Baer 2018; Rasmussen et al. 2018). The need to understand the surfaces of nanoparticles is not a qualitatively new requirement for characterization of solid-core nanoparticles, but rather an enhancement of the well-established caveats to the assumptions of stability and uniformity for nominally solid nanoparticles, which in practice are never completely stable or uniform all the way from the core to the surface (Baer et al. 2008, 2016). The effects on the surfaces of nanoparticles of the exposure to a complex environment or matrix become particularly significant for characterization of nanomaterials in the environmental (Domingos et al. 2009; Hassellöv et al. 2008), food (Linsinger et al. 2013; McClements and McClements 2013; Peters et al. 2014), and biomedical (Lynch et al. 2007; Wohlleben 2012; Wu et al. 2011; Zhang et al. 2011) contexts.

7.2.1 Definition of a Nanomaterial

EFSA guidance (EFSA et al. 2018) follows the classification and definitions from the International Organization for Standardization (ISO) to establish the vocabulary for nanomaterials, whereby *nanoscale* is defined as ranging from approximately 1 to 100 nm and *nanoparticles* are defined as objects with all three external dimensions on the nanoscale (ISO 2015); objects with only one or two external dimensions on the nanoscale are referred to as *nanoplates* and *nanofibers*, respectively. The definitive role of the size in the classification of nanomaterials is further emphasized in the definition recommended by the European Commission (EC), whereby *nanomaterial* means a natural, incidental, or manufactured material containing particles 50% or more of which in the number-size distribution have one or more external dimensions in the size range of 1–100 nm (EC 2011).

EFSA guidance, however, considers the specific cutoff at 100 nm in the above definitions to be not fully justified from a risk assessment perspective, noting that biological effects, such as toxicokinetic behavior and particle–cell interactions, "are not rigidly related to specific size thresholds" and may occur for particles that are either smaller or larger than 100 nm in size (EFSA et al. 2018). The guidance also explicitly includes materials that are not engineered as nanomaterial but contain a nanomaterial fraction as a result of manufacturing processes for powdered or particulate food. This broad interpretation of the nanomaterial definition in the food safety context effectively increases the range of sizes across which the particle size distribution needs to be determined, as measuring the fraction of particles with sizes <100 nm may not be sufficient for interpreting the results of subsequent risk assessment measurements.

7.2.2 Size

Following the above definitions, the particle size (distribution) is the first parameter that needs to be measured in order to determine whether a given material is classified as a nanomaterial. The primary conceptual challenge in implementing the size measurements in this context is the need to measure specifically the *number-size* distribution, which can be directly measured for nanomaterials only by inherently particle-counting, mainly imaging, techniques, such as electron (Hodoroaba et al. 2014; Rice et al. 2013) or scanning probe (Baalousha et al. 2014) microscopy. As the authoritative review produced by the NanoDefine project (Table 7.1) noted, such definition "was a paradigm change without metrological guidance" (Babick et al. 2016). Furthermore, the advanced as well as time- and resource-intensive nature of electron microscopy measurements often make them impractical for routine characterization of particles. Accordingly, the metrology community has attempted to investigate and validate the use of specific surface area (Hackley and Stefaniak 2013; Lecloux et al. 2017; Wohlleben et al. 2017) and solution-based (Anderson et al. 2013) measurements as proxies for obtaining particle size distributions around and below 100 nm threshold of the EC definition. Such proxy measurements evaluate the particle sizes and particle size distributions indirectly and often with size-dependent sensitivity (Anderson et al. 2013), so a calibration against the appropriate reference materials or electron microscopy measurements is typically required to validate their ability to produce a *number-size* distribution for a given material system and to establish any necessary correction factors (Babick et al. 2016).

The two-tiered approach to particle size measurements (Babick et al. 2016) recommended by the NanoDefine project (Table 7.1) should be generally applicable for implementing the EFSA guidelines (EFSA et al. 2018). The simpler *Tier 1* techniques provide estimates of particle size distributions that either clearly identify a material as a nanomaterial or reveal the need for follow-up by the *Tier 2* imaging techniques to resolve borderline or ambiguous cases.

7.2.2.1 Tier 1 Techniques for Particle Sizing: Powders

The volume-specific surface area (VSSA) measurements are the primary recommended *Tier 1* technique for materials in powder form (Babick et al. 2016). As detailed in validation studies of VSSA (Lecloux et al. 2017; Wohlleben et al. 2017), the most common implementation of VSSA relies on measuring gas adsorption, via the Brunauer-Emmett-Teller (BET) method (Hackley and Stefaniak 2013), to determine the specific surface area (SSA), which is then multiplied by the density of the material from a He-pycnometry measurement. The VSSA measurements fundamentally cannot provide information about the distribution of the particle sizes, but assuming some independent knowledge of the form-factor of particles in a material (nanoparticles, nanofibers, nanoplates), can provide an estimate of the smallest particle dimension (d_{VSSA}) that is strongly correlated (an agreement within a factor of 2 for many materials) with the median size of the number-size distribution obtained from electron microscopy (Babick et al. 2016; Lecloux et al. 2017; Wohlleben et al. 2017). This good agreement was observed for samples with 20–60% polydispersity of organic, inorganic, metal-organic, and metallic substances, porosity being one of the main caveats that required a more complex analysis procedure (Lecloux et al. 2017; Wohlleben et al. 2017). Given the definition of a nanoparticle (Section 7.2.1), d_{VSSA} effectively provides the estimate of the median particle size for a material in nanoparticle form.

For classification under the EFSA guidelines (EFSA et al. 2018), the following basic recommendations can be adapted from the VSSA validation study (Wohlleben et al. 2017). Materials with a VSSA>60 m^2/cm^3 (and thus a d_{VSSA}<100 nm) can be classified as nanomaterials. Materials with a d_{VSSA}>1000 nm (assuming spherical form-factor) can be classified as non-nanomaterials, subject to the EFSA-specific classification caveats summarized in Section 7.2.1. Materials with a d_{VSSA} between 100 and 1000 nm need to be further investigated by at least one of the *Tier 2* imaging techniques.

7.2.2.2 Tier 1 Techniques for Particle Sizing: Suspensions

Dynamic light scattering (DLS) is one of the most popular techniques for sizing particles in suspensions (Rasmussen et al. 2018), including in the context of food materials (Linsinger et al. 2013; McClements and McClements 2013). The size of colloidal particles in DLS measurements is not measured directly but estimated as the hydrodynamic equivalent diameter from their diffusion coefficient under Brownian motion. Accordingly, the size distributions obtained from DLS are intrinsically intensity-weighted, which for polydisperse materials requires specialized algorithms to extract reliable particle size distributions and can result in a few large particles dominating the signal (Anderson et al. 2013; Kato et al. 2014; Lamberty et al. 2011; Meli et al. 2012). For monodisperse samples of solid spherical reference nanoparticles, therefore, the mean diameters measured by DLS and electron microscopy typically agree very well (e.g., within 5–10% for ca. 33 nm silica particles in Lamberty et al. 2011), but in polydisperse samples—even simple mixed/multimodal ones—some of the sub-populations are not identified by DLS (Anderson et al. 2013). For realistic polydisperse samples of various size ranges in the NanoDefine survey, number-weighted medians obtained from DLS (d_{DLS}) are typically >50% larger than those from electron microscopy (d_{EM}) (Babick et al. 2016). This overestimation is typically by less than a factor of 2.5, resulting in the adjusted thresholds recommended for material classification based on DLS data: d_{DLS}<40 nm strongly indicates a nanomaterial, while d_{DLS}>250 nm implies that it may not be a nanomaterial and additional characterization is needed (Babick et al. 2016). Subject to these caveats, DLS is recommended by the NanoDefine project as a *Tier 1* technique for screening particles in suspensions (Babick et al. 2016).

Analytical centrifugation is the second class of *Tier 1* sizing techniques for suspensions recommended by the NanoDefine project (Babick et al. 2016). Centrifugation produces differential sedimentation of suspended particles based on their density and hydrodynamic mobility, which can be particularly beneficial for characterization of complex polydisperse samples (Wohlleben 2012), such as those encountered in food or biomedical applications. The two popular implementations of analytical centrifuges use disc or cuvette configurations and intrinsically measure, respectively, a scaled density function or the cumulative function of the size distribution; the use of turbidity or refractive index measurements (currently available in the cuvette configuration) to monitor the sedimentation also adds a weighting to the recorded size distribution (Anderson et al. 2013; Babick et al. 2016; Wohlleben 2012).

In multimodal suspensions of reference materials, analytical centrifugation demonstrates excellent performance in terms of separating and identifying the sub-populations in the particle size distribution, which for well-defined nanoparticles can be readily converted into number-size distributions (Anderson et al. 2013; Wohlleben 2012). The recently reported capability of analytical centrifugation to detect the presence of dimers and multimers can be beneficial for identifying the origin of such multimodal sub-populations in samples of nominally monomodal particles (Mehn et al. 2017a). For most of the more realistic polydisperse materials in the NanoDefine survey, number-weighted medians obtained from analytical centrifugation (d_{AC}) differ by <50% from d_{EM} (Babick et al. 2016; Ullmann et al. 2017). While d_{AC} values exhibit a better agreement with d_{EM} than do d_{DLS} ones, the discrepancies (including occasional major ones) between d_{AC} and d_{EM} are difficult to rationalize, so the same adjusted thresholds are recommended for material classification based on analytical centrifugation or DLS data (Babick et al. 2016).

7.2.2.3 Tier 1 Techniques for Particle Sizing: Limitations

Three potential *Tier 1* techniques were not recommended by the NanoDefine project for classification or screening measurements of particle size distributions in suspensions—particle tracking analysis (PTA), small angle x-ray scattering (SAXS), and angular light scattering (ALS)—due to limitations summarized below (Babick et al. 2016).

PTA relies on tracking the Brownian motion of individual particles based on their visualization by the light they scatter when illuminated against a dark background (Saveyn et al. 2010). Accordingly, PTA measurements at nanoscale are currently reliable only within a small range just below 100 nm (extended to smaller sizes only for strongly scattering materials) (Babick et al. 2016). Furthermore, even for larger particles in the 200–400 nm range, PTA failed to resolve populations in a multimodal sample (Anderson et al. 2013). Future improvements in the instrumentation may be able to address both the sensitivity and resolution limitations of PTA.

SAXS takes advantage of the small wavelengths of x-rays to characterize nanoscale objects and structures (Meli et al. 2012), including unique modalities for distinguishing between aggregates and constituent particles as well as roughly resolving the shape of the constituent particles (Babick et al. 2016). Another unique modality of SAXS allows the measurement of the SSA of the (constituent) particles in suspension (Babick et al. 2016; Rasmussen et al. 2018). The

effective upper limit of size measurements by SAXS, however, is about 100 nm, diminishing its usefulness for classification of nanomaterials as a stand-alone technique (Babick et al. 2016; Rasmussen et al. 2018).

ALS was originally developed for measurements of microparticles and its performance for particles in the sub-micron size range is reported to be inconsistent (Babick et al. 2016; Kuchenbecker et al. 2012). These limitations of ALS appear to be fundamental, related to relatively weak and unstructured light scattering by nanoparticles vs microparticles, so any future improvements may require the development of dedicated instruments for the submicron size range.

7.2.2.4 Tier 2 Techniques for Particle Sizing: Electron Microscopy

Both popular electron microscopy techniques—scanning electron microscopy (SEM) and transmission electron microscopy (TEM)—and their hybrids or variants (Klein et al. 2011; Meli et al. 2012; Rades et al. 2014; Rice et al. 2013) implemented across a wide range of commercial instruments are considered to be the primary *Tier 2* techniques because they provide direct measurements of the size, morphology, and aggregation state of particles across a uniquely broad size range as well as of number-size distributions from direct counting (Babick et al. 2016; Rasmussen et al. 2018). The electron microscopy size measurements produced a consistent nanomaterial classification and the best reproducibility among the methods tested in the NanoDefine project, with results falling within a factor 1.2 for half of the materials and within a factor 1.5 for most, hence the use of the number-weighted medians d_{EM} as reference values for other techniques (Babick et al. 2016).

The practical caveats of using electron microscopy for nanocharacterization are related primarily to the widely-acknowledged uncertainties and ambiguities arising from the preparation of representative samples, especially from polydisperse materials (Babick et al. 2016; Rasmussen et al. 2018). Accordingly, the use of the appropriate *Tier 1* techniques is recommended to cross-check the general plausibility of results obtained from electron microscopy (Babick et al. 2016). The fundamentals of the measurements carried out under vacuum and via exposure to high-energy electron beams also intrinsically limit the use of SEM and TEM for broad classes of sensitive samples, including most of the organic, biological, and other soft materials (Section 7.3) relevant in the context of food (Linsinger et al. 2013; McClements and McClements 2013).

7.2.3 Concentration

The concentration of nanoparticles is a parameter conspicuously absent from general discussions of their physicochemical characterization, e.g., in the overview of the OECD Testing Programme (Rasmussen et al. 2018). This may appear surprising, given the importance of quantifying the exposure or administered doses of nanomaterials in environmental (Hassellöv et al. 2008), food (Linsinger et al. 2013; McClements and McClements 2013), or biomedical (Grainger 2013; Wu et al. 2011) contexts. In a specific medical application of nanoparticles to produce magnetic hyperthermia, for example, the variations and ambiguity in specifying the concentration of nanoparticles lead to difficulties in comparing the effectiveness of treatment protocols reported by different groups (Vilas-Boas et al. 2018). For the purposes of risk assessment (EFSA et al. 2018; Linsinger et al. 2013), the dose of a nanomaterial in suspension is most commonly specified as a mass concentration (McClements and McClements 2013). But unlike in conventional toxicology or pharmacology where the known molecular weight of the specified compound allows for a trivial conversion between mass and molar concentrations, the typical heterogeneity of nanomaterials makes such nominal conversions unreliable (Hassellöv et al. 2008; Linsinger et al. 2013). Yet, the presumptive individual nature of typical nanoparticle–cell interaction events (EFSA et al. 2018; Grainger 2013; Westmeier et al. 2018; Wu et al. 2011) strongly advocates that molar concentrations or, in other words, *absolute* rather than only relative number concentrations of nanoparticles need to be considered in most applications (Shang and Gao 2014; Shard et al. 2018). Addressing the lack of a general technique for measuring concentrations of nanoparticles is the primary motivation for the InNanoPart project (Table 7.1) supported by the European Metrology Programme for Innovation and Research (EMPIR).

The ensemble techniques used to measure particle concentrations in suspensions significantly overlap with those used for particle sizing (described in Section 7.2.2.2). The signal in the ensemble techniques based on light scattering or absorption, including DLS, UV-vis absorption, and turbidimetry, fundamentally depends on both size and concentration of the particles, so the ability to reliably measure either of the two parameters strongly depends on having a monodisperse population of particles and always requires the particles to have additional properties (Shang and Gao 2014). For example, the UV-vis absorption can be used to measure particle concentration via Beer-Lambert law only for particles that exhibit specific/unique extinction peaks in UV-vis spectra and for which the molar extinction coefficient is known. Via a

combination of theoretical calculations and empirical results, the molar extinction coefficients have been derived for nanoparticles composed of common materials, including gold (Liu et al. 2007; Navarro and Werts 2013), silver (Navarro and Werts 2013), and semiconductor quantum dots (Sun and Goldys 2008; Yu et al. 2003). These molar extinction coefficients, however, are based on multiple assumptions of idealized size, composition, and internal structure, so their accuracy, and hence that of the measured particle concentrations, typically needs to be verified using independent methods (Shang and Gao 2014; Shard et al. 2018). Similarly, particle concentration measurements by turbidimetry are limited to nonabsorbing particles with known refractive index and scattering coefficient (Khlebtsov et al. 2008; Shang and Gao 2014). And DLS measurements of particle concentration, likewise, require the knowledge of refractive index and scattering coefficient for the particles being measured and for absolute concentration measurements, a calibration sample of a known concentration (Shang and Gao 2014; Vysotskii et al. 2009). For samples of nominally monomodal particles, the concentration measurements by any of the above techniques can be adjusted for the presence of dimers and multimers based on analytical centrifugation (Mehn et al. 2017a).

The single-particle techniques for measuring particle concentrations can be roughly divided in two categories: visualization and counting. The visualization techniques include PTA and electron microscopy, subject to the challenges with representative sampling analogous to those mentioned in Sections 7.2.2.3 and 7.2.2.4 (Shang and Gao 2014). The advanced counting techniques for nanoparticles in suspension typically rely on a transduction mechanism that produces a pulse for each individual particle passing by or into a detector. Resistive-pulse sensing, for example, is based on detecting a transient signal as a particle passes through a tightly constrained channel or pore (Kozak et al. 2011), but the small analysis volume and constrained flow paths raise the representative-sampling caveats, as mentioned above for the PTA, and require a standard calibration sample with a known concentration of nanoparticles for converting the pulse count into absolute concentration values (Shang and Gao 2014).

Inductively coupled plasma mass spectrometry (ICP-MS) and its variants are commonly used to analyze composition, contamination, and authenticity of food samples by detecting specific elements or atomic clusters (Taylor et al. 2018). In a standard ICP-MS measurement, a mass concentration of one or more elements can be established for nanoparticles. When analyzing a suspension of nanoparticles by ICP-MS with sufficient temporal resolution, instead of the

conventional quasi-constant flow of ions, bunches of ions (peaks in ion intensity) corresponding to individual particles are detected, resulting in single-particle ICP-MS (spICP-MS) technique, for which applications to nanoparticles in complex media are being explored in the environmental and biomedical contexts (Montaño et al. 2016). While spICP-MS features an advantage of a signal proportional to the masses of individual particles (or aggregates), which helps to resolve some ambiguities associated with other counting techniques, accurate measurements of absolute number concentrations would require either a perfect transport and atomization/ionization in plasma, or appropriate calibration standards to account for instrumental factors (Montaño et al. 2016; Shang and Gao 2014).

7.2.4 Composition

For nanoparticles in suspension, the most general techniques recommended for elemental analysis are inductively coupled plasma optical emission spectrophotometry (ICP-OES), ICP-MS, and their variants (Montaño et al. 2016; Rasmussen et al. 2018; Taylor et al. 2018). The specific variant optimal for characterization of a given nanomaterial needs to be selected based on the requirements, for example, ICP-MS is generally more sensitive than ICP-OES but performs poorly for light elements, whereas ICP-OES is suitable for both organic and inorganic nanomaterials but may be difficult for complex samples due to the interference between emission lines of different elements (Rasmussen et al. 2018; Taylor et al. 2018). The spICP-MS variant can be used to statistically sample the elemental composition of individual nanoparticles (Montaño et al. 2016).

For samples of powder or dried nanoparticles, energy dispersive x-ray spectroscopy (EDX) implemented in both SEM and TEM instruments can provide a detailed analysis of elemental composition, even for individual particles (Hassellöv et al. 2008; Rades et al. 2014; Rasmussen et al. 2018). EDX, in principle, is suitable for detecting elements with an atomic number >5, whereas for lighter elements and for resolving chemical states of some elements electron energy loss spectroscopy (EELS) can be used as a complementary or alternative technique (Hassellöv et al. 2008; Rasmussen et al. 2018). For sufficiently small nanoparticles (ca. <10 nm), preferably of uniform composition, x-ray photoelectron spectroscopy (XPS) can provide detailed information about the elemental composition and the chemical states of those elements (Baer and Engelhard 2010; Baer et al. 2010), in many cases with high sensitivity to the presence of minor elements

(Shard 2014; Shard and Clifford 2017). With the help of models and simulations, XPS data can be also interpreted to check the internal structure of core-shell nanoparticles (Cant et al. 2016; Chudzicki et al. 2015; Powell et al. 2016, 2018; Shard 2012).

7.2.5 Surface

The chemistry of the particle surface is regulatory relevant information for nanomaterials in general (Rasmussen et al. 2018) and is specifically of interest in the context of food (EFSA et al. 2018; McClements and McClements 2013) and biomedical (Grainger 2013; Lynch et al. 2007; Wu et al. 2011) applications. Nanomaterials inherently have a much larger specific surface area than do bulk materials, so surfaces play a critical role in the interactions of nanoparticles with their environment and with each other (Wu et al. 2011). Furthermore, surfaces of nanoparticles can be intentionally or unintentionally functionalized, whereby the intentional functionality may modify the stability of the particles or their affinity for the matrix or other objects, whereas the unintentional functionalization or modification typically occurs due to specific or nonspecific interactions with the environment (Baer 2018).

The most common techniques for surface characterization of nanoparticles are XPS and time-of-flight secondary ion mass spectrometry (ToF-SIMS), both techniques are very sensitive and inherently surface-specific, with typical sampling depth of ca. 10 nm and 1–2 nm, respectively (Baer et al. 2008, 2010; Rafati et al. 2016; Rasmussen et al. 2018; Shard 2014). The shallow sampling depth makes XPS and ToF-SIMS particularly suitable for chemical analysis of monolayers of organic or biological molecules as well as a-few-nm-thin polymeric or inorganic surface layers that provide functionality (e.g., capping/passivation, electronic-structure confinement, etc.) or interact with the environment (e.g., via oxidation, protonation, or hydration). The combination of the sampling depth and angle-dependence of the photoelectron signal enable the use of XPS for characterization of shells in core-shell nanoparticles (Cant et al. 2016; Chudzicki et al. 2015; Powell et al. 2016, 2018; Shard 2012).

Adapting XPS to characterization of complex materials on nanoparticles typically starts with a detailed analysis of model (mono)layers on flat surfaces, e.g., for organic molecules (Techane et al. 2011), polymers (Lock et al. 2010), DNA (Petrovykh et al. 2004), peptides (Fears et al. 2013), or proteins (Ray and Shard 2011; Ray et al. 2015), followed by the extension and validation of the methodology to the analogous (mono)layers on nanoparticle surfaces (Belsey et

al. 2015, 2016; Chudzicki et al. 2015; Minelli et al. 2014; Minelli and Shard 2016; Rafati et al. 2016; Shard 2012; Techane et al. 2011).

Notably, molecular adsorption or attachment onto surfaces of model nanoparticles can be followed quantitatively by a variety of individual or complementary sizing techniques, for example, by DLS, PTA, analytical centrifugation, and UV-vis spectroscopy (Bell et al. 2013; Belsey et al. 2015), by DLS, SAXS, and analytical centrifugation (Minelli et al. 2014), by DLS, SAXS, PTA, and analytical centrifugation (Gollwitzer et al. 2016), or by analytical centrifugation (Davidson et al. 2017). Effects of the environment and surface adsorption on the surface charge of nanoparticles can be also followed by several complementary techniques, including electrophoretic light scattering (Lamberty et al. 2011), PTA, and resistive-pulse sensing (Sikora et al. 2015). Quantitative measurements of surface adsorption onto more realistic particle populations and from more complex media, however, remain an important but unresolved analytical challenge (Hassellöv et al. 2008; Linsinger et al. 2013; Lynch et al. 2007; McClements and McClements 2013; Wu et al. 2011; Zhang et al. 2011).

7.3 Soft Nanoparticles

The regulatory consideration of soft nanoparticles to-date can be characterized as equivocal. The original EC Recommendation for a definition of a nanomaterial (EC 2011) refers to a particle as a minute piece of matter with defined physical boundaries. This terminology neither explicitly includes or excludes soft particles, so amendments have been proposed to resolve this ambiguity, with one option specifying exclusively “solid matter” and another option that would include soft materials, such as micelles and liquid droplets (Rauscher et al. 2015). EFSA guidance resolves the regulatory ambiguity in the food context by including within the scope “[n]anoscale entities made of natural materials that have been deliberately produced to have nano-enabled/enhanced properties, or that have been modified for use in the development of other nanoscale materials, e.g. for encapsulating (bioactive) compounds” and “organic nanomaterial, such as encapsulates” (EFSA et al. 2018). The defining characteristic of this scope appears to be the deliberately produced or engineered nature of the nanomaterial, as it excludes “other ‘natural’ nanoscale entities [that] may be present in food/feed” (EFSA et al. 2018). This explicit consideration of the soft nanomaterials by EFSA is eminently justified in the food context because food-grade nanoparticles are commonly produced from soft materials, such as proteins, carbohydrates, and

lipids, and by methods that inherently produce soft particles: homogenization, antisolvent precipitation, spontaneous emulsification, and coacervation (McClements and McClements 2013).

7.3.1 Visualization of Particle Morphology

Examples of TEM imaging of soft nanoparticles tend to be primarily from the biomedical rather than food context, but they provide good illustrations of the state-of-the-art implementations of TEM for this general class of samples. The first overarching conclusion from comparative studies of different preparation approaches of soft nanoparticles for TEM is that drying such samples produces a broad range of artifacts, for example, as illustrated by systematic imaging of liposomes (Franken et al. 2017) and polymeric micelles (Patterson et al. 2015) or by observing the destruction of vesicles in a mixed population of polymeric vesicles and micelles (Laan and Denkova 2017). Accordingly, dried samples of soft nanoparticles are not suitable for obtaining TEM images that are representative of the properties of the population being characterized. In contrast, cryogenic TEM (cryo-TEM) is considered to be much more reliable in preserving the morphology of soft particles and in protecting them from damage by the high-energy electron beam used during the imaging (Franken et al. 2017; Klang et al. 2012; Laan and Denkova 2017). The morphology and microstructure have been successfully imaged by cryo-TEM for different liposome variants (cubosomes, hexosomes, micellar cubosomes), transitional mesophases during formation of liposomes, vesicles, amphiphilic Janus dendrimers, spherical dendrimersomes, spherical and wormlike micelles, and microemulsions (Helvig et al. 2015). Nanomedicine drug carriers successfully imaged by cryo-TEM include liposomes, colloidal lipid emulsions, solid lipid nanoparticles, thermotropic and lyotropic liquid crystalline nanoparticles as well as polymer-based colloids and delivery systems for nucleic acids (Kuntsche et al. 2011). While all of these examples used model systems exhibiting varying degrees of realistic features, the soft particles considered for food (McClements and McClements 2013) or biomedical (Klang et al. 2013) applications include many lipid and liquid-crystalline nanoparticles as well as microemulsions, the morphology and microstructure of which can be investigated following analogous cryo-TEM methodologies.

Among SEM variants, environmental SEM (ESEM) helps to avoid some of the drying artifacts by imaging soft particles under a humid atmosphere and thus in a partially hydrated

state (and following the kinetics of dehydration) or even in emulsions (Stokes 2001) and liquid droplets (Méndez-Vilas et al. 2009). Cryogenic SEM (cryo-SEM) can be used for morphological characterization of soft materials: compared to cryo-TEM, it reveals partial 3D information about the shapes of soft nanoparticles, e.g., cubosomes, hexosomes, or nanoemulsions, but does not provide details of their internal structure (Boyd et al. 2007; Klang et al. 2012, 2013).

7.3.2 Sizing of Soft Particles by Electron Microscopy

The fundamental importance of electron microscopy for characterization of solid nanoparticles is difficult to overstate: it is the most reliable method for sizing solid nanoparticles and provides reference data on sizes and size distribution for benchmarking and validation of other techniques (Section 7.2.2). In contrast, soft nanoparticles are not easily considered following the strict size-threshold approach to definition of solid nanoparticles because their sizes generally depend on the chemical and physical forces (and history of such forces) exerted by their environment (EFSA et al. 2018; Rauscher et al. 2015). The discretionary interpretation of the threshold size for nanomaterials in the EFSA guidance (EFSA et al. 2018) provides a more natural framework for incorporating the unique challenges encountered in characterization of soft particles.

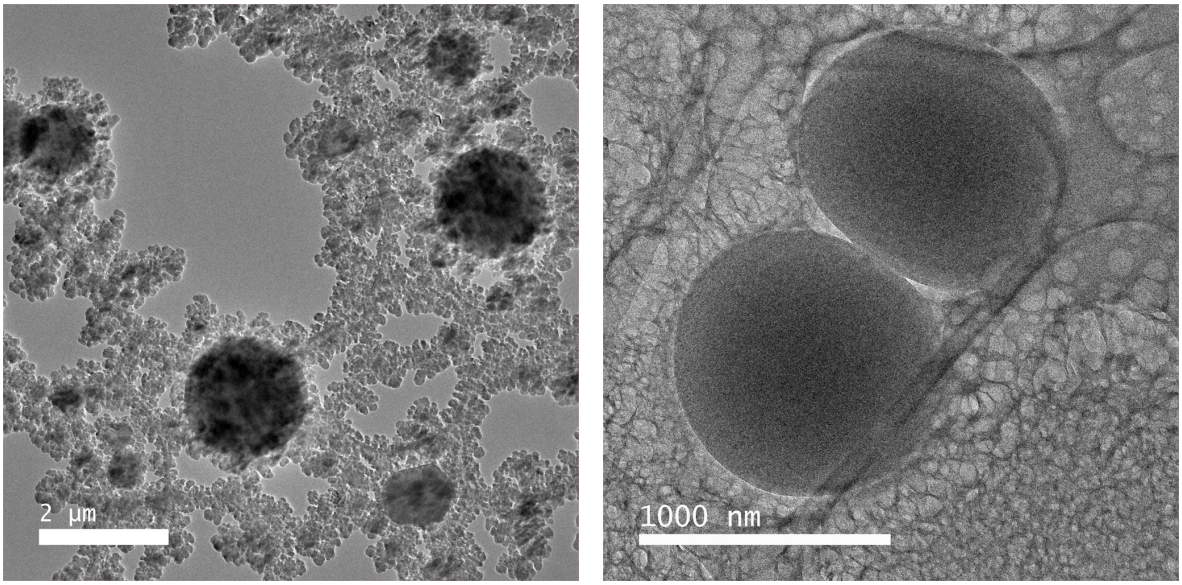

Figure 7.1 *Staphylococcus aureus* bacterial cells imaged by cryo-TEM. Both samples have been prepared by robotic plunge-freezing in liquid ethane, the only difference in the preparation prior to freezing was the medium in which the live cells have been resuspended: deionized water (left) and 40% dextran in 0.01 M phosphate-buffered saline (PBS) (right).

An important insight into the size measurements for soft particles by electron microscopy is provided by considering another class of model systems: bacteria and other microorganisms. *Staphylococcus aureus* (*S. aureus*) bacteria are a particularly convenient model system for size measurements due to their nearly spherical shape (Monteiro et al. 2015; Zhou et al. 2015) and robust viability and mechanical stability under a wide range of physicochemical conditions (Monteiro et al. 2015; Sousa et al. 2015). As illustrated in Figure 7.1, different preparation protocols strongly affect the apparent sizes and size distributions of *S. aureus* cells imaged by cryo-TEM: images from either of the two preparations interpreted on their own would provide very different conclusions regarding both polydispersity and mean particle sizes in the typical *S. aureus* population.

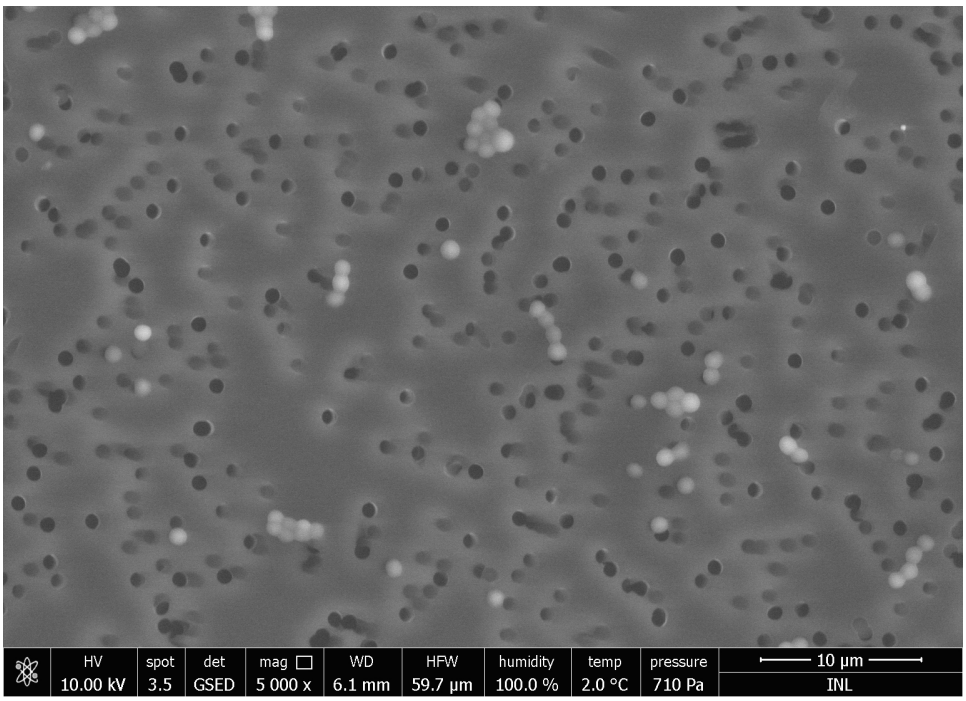

Figure 7.2 *Staphylococcus aureus* bacterial cells imaged by ESEM. Live cells were filtered from suspension onto a polycarbonate filter with ca. 0.2 µm pores, which appear in the image as dark circular features. A piece of wet filter with cells was introduced into the ESEM chamber and imaged with a 10-kV beam and a gaseous secondary electron detector (GSED), on a Peltier stage at 2°C and 100% relative humidity at the background gas pressure of 710 Pa.

By analogy with lipid nanoparticles (Boyd et al. 2007; Klang et al. 2013), ESEM should be a good choice of a complementary and self-consistent electron microscopy technique for cross-validation of cryo-TEM results. Furthermore, reported evidence indicates that under ESEM

conditions microorganisms can even remain viable (Ahmad et al. 2011; Misirli et al. 2007; Ren et al. 2008), suggesting that the environmental (including physicochemical) stress induced under ESEM conditions is relatively mild and thus, hypothetically, may not strongly affect the size of the imaged soft particles.

The apparent size of the *S. aureus* cells imaged by ESEM (Figure 7.2) is ca. 1.1 μm, in good agreement with that in the cryo-TEM image prepared using the second method in Figure 7.1. When fixed and dried *S. aureus* cells are imaged by SEM under vacuum, their apparent sizes are typically in the 0.6–0.8 μm range (Monteiro et al. 2015; Sousa et al. 2015; Zhou et al. 2015), so at least some drying clearly can be avoided under both ESEM and cryo-TEM conditions. In high-resolution optical microscopy images, acquired in liquid using either phase-contrast or structured-illumination mode, the individual live *S. aureus* cells consistently appear to be larger than 1 μm and as large as 1.5 μm (Monteiro et al. 2015; Zhou et al. 2015). These variations clearly illustrate the strong dependence of the size of a soft particle on its environment, as postulated at the beginning of this section. The ca. 0.5–1.5 μm size range observed for *S. aureus* model also is close to the limit for a systematic comparison between the optical and electron microscopy techniques, as optical microscopies become resolution-limited (Yao and Carballido-López 2014) in the ca. 100 nm "nanoscale" range formally defined for nanomaterials (Section 7.2.1).

7.3.2.1 Electron Microscopy in Liquid Environment

Considering the functional aspects of the nanomaterial definition in the EFSA guidance (EFSA et al. 2018), which suggests that the relevant size range or threshold can be established based on phenomena such as nanoparticle–cell interactions (Gioria et al. 2018; Grainger 2013; Westmeier et al. 2018), the most practically relevant size of a soft particle should be defined and measured under the conditions that are as close as possible to those of the intended use. For most of the soft particles in food (McClements and McClements 2013) or biomedical (Gioria et al. 2018; Grainger 2013) applications, the relevant environment is that of being a component in a mixed suspension of other particles in a complex media. Therefore, even the most reductionist version of the relevant environment would imply size measurements of soft particles in an aqueous solution. There are three major variants of TEM that are able to measure nanoparticles in

solution, implementing each of them requires specialized sample cells and modifications of the standard instruments.

In situ liquid TEM is implemented using a liquid sample cell that can be introduced into a TEM instrument; in such a sample cell a thin (50–500 nm) layer of liquid is confined between ultra-thin (ca. 50 nm) membranes of "electron transparent" material, such as silicon nitride or graphene. For model Pt-core polymeric micelles, in which the Pt provided intrinsic staining, *in situ* liquid TEM imaging revealed a size distribution having a similar mean at ca. 90 nm and a somewhat narrower width compared to cryo-TEM imaging; in dry-state TEM the distribution was shifted to smaller sizes, while with uranyl acetate staining a distinct second population emerged, centered about 45 nm (Patterson et al. 2015). The frame rate in that implementation of *in situ* liquid TEM enabled real-time observation of particle agglomeration (Patterson et al. 2015).

Using a similar type of liquid sample cell, a scanning TEM (STEM) variant can be implemented to take advantage of the atomic number (Z) contrast intrinsic to STEM and to be able to measure, e.g., gold nanoparticles with diameter of ca. 1.4 nm, through water layers up to 3 μm in thickness (de Jonge et al. 2010). The intrinsically slower frame rate in STEM compared to TEM imaging limits the temporal resolution for dynamic measurements in this configuration, but "video rate" with 10 μs per pixel dwell time has been demonstrated for tracking the movement of 5-nm gold nanoparticles (Ring and de Jonge 2012).

Another implementation of STEM imaging takes advantage of ESEM instruments and their capability to operate at 100% relative humidity to perform STEM measurements through a drop of liquid placed on a conventional TEM grid (Bogner et al. 2005, 2007). These "wet STEM" measurements have demonstrated for gold and latex nanoparticles as well as for liquid emulsions of styrene or polystyrene–styrene in water; latex nanoparticles with different surface modification could be distinguished as well (Bogner et al. 2005).

All the above specialized variants of TEM for measurements in liquid are currently implemented on a limited number of instruments, so until a broader adoption and commercialization of these techniques, they would not be practical for routine characterization, even as the equivalent of *Tier 2* techniques for soft particles. In the meantime, one approach for amplifying the impact of the few existing instruments would be to use them to validate the

methodology (both preparation and imaging of specific soft particle systems) for the more commonly available cryo-TEM.

7.3.3 Ensemble Techniques for Sizing Soft Particles

Among the ensemble techniques for characterizing suspensions of soft nanoparticles, flow field-flow fractionation (FFFF or F4) and its many variants are commonly used in the nanomedicine context (Qureshi and Kok 2011; Wagner et al. 2014; Zattoni et al. 2014). The FFFF fundamentally is a separation technique based on particle diffusion coefficients (Wagner et al. 2014), which makes it suitable for separation of particles in a broad size range from nanometers to micrometers; FFFF has been recently approved by the US Food and Drug Administration (FDA) for validation of protein drug samples (Zattoni et al. 2014). FFFF is often coupled to an optical detection technique, such as static multi-angle light-scattering (MALS) (Zattoni et al. 2014) or DLS (Mehn et al. 2017b; Sitar et al. 2017), to measure size distribution analysis of soft particles and to investigate their aggregation in (native) solution conditions. In the context of drug-delivery soft nanoparticles, FFFF-MALS has been successfully used for characterization of lipid and (bio)polymer particles (Wagner et al. 2014; Zattoni et al. 2014).

In a systematic comparison of different sizing techniques for monodisperse samples of empty and drug-loaded liposomes, both conventional DLS and FFFF-DLS measurements measured their mean sizes as 85 and 80 nm, respectively; both values agreed with those from analytical centrifugation (Mehn et al. 2017b) and PTA (Gioria et al. 2018) measurements. In contrast, average diameters of 63 and 57 nm, respectively, were determined for the empty and drug-loaded liposomes by cryo-TEM (Mehn et al. 2017b), indicating a strong bias between the values measured by electron microscopy and those obtained from the ensemble techniques in solution. After incubation with serum proteins, conventional DLS indicated a shift to a smaller size, however, essentially identical elution profiles in FFFF-DLS demonstrated that for these model particles protein adsorption from serum was negligible and the indication from conventional DLS was misleading (Mehn et al. 2017b). For polydisperse samples, DLS was found to be unable to resolve the complex particle size distributions that were successfully resolved by both PTA and FFFF-DLS (Mehn et al. 2017b).

7.4 Reproducibility in Nanocharacterization

Reproducibility challenges associated with the characterization of nanoparticles arise directly from many of the same inherent characteristics that make them of interest in applications (Baer 2018). The multidisciplinary nature of the communities involved in producing and characterizing nanoparticles further compounds the difficulties of communicating and understanding the underlying phenomena, best practices, nuanced interpretation of the characterization data, and particularly of any associated health or environmental risks (Baer 2018; Nel et al., 2015; Petersen et al., 2014). Outlined below are some of the major issues that have been identified by expert communities as contributing to the reproducibility challenges in nanocharacterization.

Reproducibility in the synthesis and production of nanomaterials is notoriously difficult to ensure. Nominally the same protocols, even when applied by the same practitioner, will often produce nanomaterials with batch-to-batch variations at both short- and long-term timescales (Baer 2018). In some cases, extensive characterization of the produced nanomaterial may reveal the factors that contribute to this variability, but the effort and expertise required for such comprehensive characterization make it impractical to be pursued routinely.

Nanomaterials are fundamentally dynamic systems, so over time they can be dramatically affected by internal thermodynamically-driven processes and by the environmental conditions and other external interactions during storage, transport, handling, and even preparation for characterization (Baer 2018; Baer et al. 2018; McClements and McClements 2013; Petersen et al., 2014). Collecting and analyzing the detailed provenance information along with carefully designing and implementing appropriately-timed characterization plans are the most important measures recommended to address this challenge (Baer 2018; Baer et al. 2016, 2018).

Both extraction from and dispersion in the matrix are critically important for handling and characterization of nanomaterials (Baer et al. 2018; McClements and McClements 2013; Petersen et al., 2014; Rasmussen et al. 2018). Some of the steps in such complex protocols may have different effects on nanomaterials compared to those assumed based on the standard procedures used for small molecules, polymers, or powders and microparticles.

Adapting the traditional toxicology and toxicity assays for assessment of health risks associated with nanoparticles requires a careful review and validation of the underlying principles and mechanisms, ranging from understanding the exposure dose, to chemical (e.g.,

impurities from synthesis) and biological (e.g., endotoxin) contamination, to the effects of nanoparticles on the assay read-out (e.g., interference or quenching in colorimetric assays) (Grainger 2013; McClements and McClements 2013; Nel et al., 2015; Petersen et al., 2014).

7.5 Concluding Remarks

The critical importance of physicochemical characterization of nanomaterials is emphasized in the regulatory guidelines and requirements for their food and medical applications (EFSA et al. 2018; Pita et al. 2016; Rasmussen et al. 2018). Since the EC issued the original recommended definition of a nanomaterial (EC 2011), a significant concerted effort by various European authorities, metrology organizations, international standards bodies, and major international projects resulted in a dramatically improved understanding of nanocharacterization needs and in the development and validation of characterization methods for nanomaterials. In particular, the importance of particle sizes and size distribution for classification of nanomaterials has generated a wave of metrological and analytical activity on improving and validating the sizing methods for solid nanoparticles (Babick et al. 2016; Rasmussen et al. 2018). The characterization of composition and surface properties of nanoparticles continues to improve in terms of speed, sensitivity, and reliability, based on adapting and extending analytical methods from the more traditional materials and surface analysis domains (Baer et al. 2010; Rasmussen et al. 2018). Measurement of absolute particle concentrations has received relatively less direct attention to-date, despite its practical importance for the current and future applications of nanoparticles, but it benefits indirectly from the development of new and improved particle sizing techniques that can be adapted for concentration measurements. Finally, characterization of soft particles is an area of recognized importance and the resulting growth in analytical techniques and capabilities.

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