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Standardization of Nanoparticle Characterization: Methods for Testing Properties, Stability and Functionality of Edible Nanoparticles

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**Standardization of Nanoparticle Characterization: Methods for Testing Properties,
Stability and Functionality of Edible Nanoparticles**

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ABSTRACT

There has been a rapid increase in the fabrication of various kinds of edible nanoparticles for oral delivery of bioactive agents, such as those constructed from proteins, carbohydrates, lipids, and/or minerals. It is currently difficult to compare the relative advantages and disadvantages of different kinds of nanoparticle-based delivery systems because researchers use different analytical instruments and protocols to characterize them. In this article, we briefly review the various analytical methods available for characterizing the properties of edible nanoparticles, such as composition, morphology, size, charge, physical state, and stability. This information is then used to propose a number of standardized protocols for characterizing nanoparticle properties, for evaluating their stability to environmental stresses, and for predicting their biological fate. Implementation of these protocols would facilitate comparison of the performance of nanoparticles under standardized conditions, which would facilitate the rational selection of nanoparticle-based delivery systems for different applications in the food, health care, and pharmaceutical industries.

Keywords: nanoparticles; nanoemulsions; characterization; analysis; standardization.

INTRODUCTION

The development of edible nanoparticles constructed from food-grade components has rapidly grown in recent years for various applications in the food, health care, and pharmaceutical industries: encapsulation, protection and delivery of active agents; texture modification; and control of optical properties (Acosta, 2009; Sanguansri and Augustin, 2006; Velikov and Pelan, 2008). Concurrently, there has been a major research effort directed towards understanding and mitigating the potential toxicity of edible nanoparticles (Acosta, 2009; Borel and Sabliov, 2014; McClements, 2013; McClements and Xiao, 2012; Raynes et al., 2014). As a result of these research efforts, there has been a sharp increase in the number of publications on “nanoparticles” and “food” during the past decade (**Figure 1**). At present, there is no consensus in the literature about the precise size range that should be used to define a particle as a nanoparticle (McClements and Rao, 2011; Raynes et al., 2014). Some researchers have reserved this term for particles with diameters smaller than 100 nm, whereas others have used it to refer to particles less than 1000 nm or even other values between these two limits. However, government organizations, such as the European Commission, have defined nanoparticles as being in the size range 1 to 100 nm (Bleeker et al., 2013). Ideally, we recommend that researchers reserve the term “nanoparticle” to refer to particles with diameters less than 100 nm, but given that in practice researchers use this term more broadly, we have also considered particles less than 1000 nm in this article.

Food-grade nanoparticles may be fabricated from various building blocks (such as proteins, carbohydrates, lipids, and minerals) using a number of different fabrication methods (such as homogenization, grinding, antisolvent precipitation, spontaneous emulsification, and

coacervation) (McClements, 2014). This diversity of building blocks and fabrication methods leads to nanoparticles with different physicochemical properties and functional attributes. For example, nanoparticles may vary in their composition, structure, charge, dimensions, physical state, and digestibility (**Figure 2**). Selected examples of different types of food-grade nanoparticles that have recently been reported in the literature are listed in **Table 1**.

At present there is no standardized protocol for characterizing the properties and functional performance of food-grade nanoparticles that is widely accepted by researchers in the food and other industries. Instead, different research groups use different analytical tools and testing methods to characterize the nanoparticles they have produced. This makes it difficult to compare the relative merits and limitations of different kinds of food-grade nanoparticles for specific applications. The purpose of this article is to review the different analytical instruments and protocols available to characterize nanoparticles, and to propose standardized testing protocols to characterize their properties and performance. Adoption of these protocols would enable researchers from different research groups to compare their results in a more systematic and meaningful manner, and would help manufacturers of commercial products to select the most appropriate nanoparticles for particular applications. This article should be particularly useful for those researchers who are starting to utilize food-grade nanoparticles as delivery systems, and need a background in their characterization.

CHARACTERIZATION OF NANOPARTICLE PROPERTIES

After a batch of food-grade nanoparticles has been manufactured it is important to thoroughly characterize their structural and physicochemical properties (**Figure 2**). In this

section, we briefly review some of the most widely used analytical methods and protocols for isolating and characterizing nanoparticle properties.

Isolation

Food-grade nanoparticles may be present in various types of matrices after they have been prepared depending on the constituents and fabrication method used, *e.g.*, dispersed within simple aqueous solutions, embedded within glassy matrices in powders, or suspended within complex multicomponent matrices. In addition, they may be the only type of particulate matter present, or they may be mixed with other types of particles or polymers. In simple systems, such as nanoparticles dispersed within an aqueous solution, it is possible to directly analyze their characteristics without much sample preparation. In more complex systems, it is sometimes possible to obtain information about nanoparticle properties without isolating them from the original matrix (*e.g.*, using certain types of microscopy), but in many cases it is necessary to isolate them from the other components in the matrix prior to analysis (Singh et al., 2014). This is particularly true when the matrix contains other types of particles or polymers that would interfere with the analysis of nanoparticle properties.

The method used to isolate nanoparticles from their environment is highly dependent on the precise nature of the system being characterized, and has to be carefully worked out on a case-by-case basis (Szakal et al., 2014). Nevertheless, some procedures are listed below that may be useful for a variety of different applications:

Matrix disruption: If nanoparticles are present within a solid or semi-solid matrix, then it may be necessary to breakdown the matrix and suspend the nanoparticles released within an appropriate aqueous or organic solvent prior to analysis (Szakal et al., 2014). For powdered

nanoparticles, this may simply be achieved by dispersing the powder within a suitable solvent, provided all the matrix components are fully soluble within the solvent. For example, hydrophobic protein (zein) nanoparticles that have been converted into a powdered form by spray drying can simply be dispersed in water prior to analysis (Chen and Zhong, 2014). For nanoparticles dispersed within semi-solid food matrices, this may be achieved using mechanical devices (such as high speed blenders), chemical treatments (such as acid or alkali), and/or enzyme treatments (such as proteases, amylases, or lipases) (Szakal et al., 2014; Szalai et al., 2014). Chemical or enzymatic treatments may also be useful for breaking down other types of particles or polymer in the matrix (provided they do not alter the properties of the nanoparticles being analyzed). Nanoparticles may then be isolated from the other components in the disrupted matrix based on differences in their density, size, or stability.

Gravitational separation: If nanoparticles are present within a fluid medium that contains other types of particulate or polymeric matter (such as oil droplets, air bubbles, protein aggregates, or polysaccharide molecules), then it may be possible to separate the nanoparticles based on gravitational separation. The velocity of gravitational separation in a dilute system is given by the following equation:

$$U = -\frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1} \quad (1)$$

Here, U is the velocity of particle movement (+ for creaming; - for sedimentation), g is the acceleration due to gravity, r is the particle radius, ρ is the density, η is the shear viscosity, and the subscripts 1 and 2 refer to the surrounding liquid and particles, respectively. More

sophisticated models are available that take into account polydispersity, non-spherical particles, particle fluidity, particle-particle interactions, and non-Newtonian fluids (McClements, 2005).

This equation shows that the rate of gravitational separation depends on the density contrast between the particles and surrounding liquid, the particle size, and the continuous phase viscosity. Nanoparticles typically have a very small particle size and therefore move very slowly due to gravity. It may therefore be possible to separate them from other particulate components by simply letting them stand for a certain time: large particles may rapidly cream or sediment, leaving the small nanoparticles. In some cases it may be necessary to dilute the system first so as to reduce the viscosity of the surrounding fluid or to prevent particle-particle interactions inhibiting separation.

Centrifugation: This approach is similar to the one based on gravitational separation, except that a centrifugal force is used to speed up particle movement rather than simply relying upon weak gravitational forces. Equation 1 can still be used to calculate the particle velocity, but the gravitational acceleration (g) is replaced by the centrifugal acceleration ($\omega^2 R$), where ω is the angular velocity and R is the rotor axis. Centrifugation can be used to separate nanoparticles from other particles or polymers that have different densities or dimensions: particles denser than the surrounding solvent move downwards, whereas those less dense move upwards; large particles move more rapidly than smaller ones.

Centrifugation may also be useful for separating nanoparticles from a surrounding liquid that contains dissolved materials that interfere with particle characterization, such as acids, bases, buffers, minerals, or polymers. In this case, high speeds may be needed to separate the small nanoparticles from the surrounding liquid (since $U \propto r^2$), with the actual value depending on the

magnitude of the density contrast ($\rho_2 - \rho_1$) for the nanoparticles involved.

Dialysis and filtration: Nanoparticles may be separated from other components in a liquid based on their ability to selectively penetrate through pores with different sizes: solutes, polymers, or particles appreciably smaller than the pores will easily travel through, whereas those that are larger will be retained. This phenomenon can be used to separate nanoparticles from smaller or larger substances within a complex mixture by dialysis, filtration, or ultrafiltration (**Figure 3**). For example, nanoparticles can be separated from small solutes or polymers by using a semi-permeable membrane with pores appreciably smaller than the nanoparticles (but larger than the other components). Alternatively, nanoparticles can be separated from large particles or polymers using a membrane with pores appreciably larger than the nanoparticles (but smaller than the other components).

Selective precipitation: Nanoparticles can sometimes be separated from other components in the surrounding liquid based on differences in their relative aggregation stabilities. Conditions such as pH, ionic strength, solvent quality, or temperature can be altered so as to selectively precipitate either the nanoparticles or the components within the surrounding solution (**Figure 4**). The precipitated phase can then be removed by filtration, sedimentation, or centrifugation.

In all cases, it is critical that the properties of the nanoparticles are not altered by the isolation and purification procedure used. In the remainder of this article we will assume that the fabricated nanoparticles are dispersed within a simple aqueous solution.

Particle composition

Carrier Matrix

Food-grade nanoparticles may be fabricated from a range of different food ingredients,

including lipids, proteins, carbohydrates, and minerals (McClements, 2014). The manufacturer of a batch of nanoparticles usually has good knowledge about the types and concentrations of ingredients used to assemble them, and may therefore be able to infer their composition. However, for certain types of nanoparticles this inference may not be possible. For example, a manufacturer may fabricate nanoparticles based on complex coacervation by mixing a protein and a polysaccharide solution together, but not know the relative proportion of proteins and polysaccharides present in the final particles (since some of them may remain in the surrounding liquid). Alternatively, one may form lipid nanoparticles by homogenization of an oil phase with an aqueous phase containing a surface-active protein, but not know how much of the protein actually adsorbs to the nanoparticle surfaces and how much remains in the aqueous phase. In these cases, it may be important to measure the actual composition of the nanoparticles formed. Typically, the nanoparticles would first have to be separated from the surrounding matrix using the methods described in the previous section, such as sedimentation, centrifugation, filtration, dialysis, or selective precipitation. They would then be dispersed in a suitable solvent, such as distilled water or buffer solution. Analytical tools capable of measuring the concentration of specific components in the nanoparticles could then be used to measure the overall particle composition (Nielsen, 2010; Singh et al., 2014). An alternative indirect approach to determine the amount of a specific component within a nanoparticle involves measuring the amount that has not been incorporated into the nanoparticles *i.e.*, that remaining in the surrounding fluid. For example, dialysis, filtration, or centrifugation could be used to isolate the nanoparticles from the surrounding fluid, and then the amount of the specific component in the surrounding fluid could be measured using a suitable analytical method. The amount of the component (m_{NP}) in the

nanoparticles could then be estimated from the total amount present (m_{Total}) minus the amount in the surrounding fluid (m_{SF}): $m_{\text{NP}} = m_{\text{Total}} - m_{\text{SF}}$. A list of different analytical methods for providing information about nanoparticle composition is highlighted in **Table 2**. The most suitable method for a particular application depends on the precise nature of the nanoparticles, *i.e.*, the type and amount of the different constituents present. A brief summary of some common analytical methods that could be used to measure nanoparticle composition is given below, and more detailed information can be found in the literature (Nielsen, 2010).

The total protein content of nanoparticles may be obtained by methods based on nitrogen analysis (such as Kjehldahl or Dumas methods) or spectroscopy (such as direct, Biuret, or Lowry methods). The type and amount of different kinds of proteins present within a nanoparticle can be obtained using electrophoresis (*e.g.*, SDS-PAGE), size exclusion chromatography, mass spectrometry, and some biochemical methods (such as enzyme or antibody assays). The total lipid content of nanoparticles can be measured by various solvent or non-solvent extraction methods, such as the Soxhlet, Goldfish, Babcock, Gerber, and detergent methods. Information about the type and amount of specific lipid components present can be obtained using thin layer chromatography (TLC), gas chromatography (GC), or high performance liquid chromatography (HPLC). In certain cases it is possible to obtain *in situ* information about lipid type and amount using spectroscopy methods, such as UV-visible, infrared, NMR, or Raman spectroscopy. The total carbohydrate content of nanoparticles can be measured using wet chemistry approaches, such as colorimetric, gravimetric, or titration methods. More detailed information about specific carbohydrate types and amounts can be obtained using chromatography (TLC, GC or HPLC), electrophoresis, or biochemical methods (such as enzyme-based assays) (Singh et al., 2014). The

total mineral content of nanoparticles can be obtained by ashing, whereas information about the type and concentration of specific minerals present can be determined by wet chemistry, mass spectrometry, spectroscopy (atomic absorption or emission), and X-ray methods (Singh et al., 2014). Information about the interactions between different components within nanoparticles can be obtained using spectroscopy methods such as infrared and Raman (Cho et al., 2014; Zou et al., 2012).

Active Ingredients: Loading, Encapsulation, Retention, and Release

Food-grade nanoparticles are commonly developed to encapsulate, protect, and release active ingredients, such as nutraceuticals, vitamins, pharmaceuticals, flavors, colors, and preservatives. It is therefore important to have analytical tools that can determine the concentration and location of active ingredients within nanoparticle suspensions. A nanoparticle-based delivery system should have a number of characteristics if it is going to be suitable for encapsulating active ingredients: high loading capacity (LC), high encapsulation efficiency (EC), and high retention efficiency (RE).

The *loading capacity* is defined as the maximum amount of active ingredient that can be loaded into the nanoparticles per unit mass of carrier particle:

$$LC = 100 \times m_A / m_P = 100 \times c_A / c_P \quad (2)$$

Here, m_A and m_P are the total masses of the active ingredient and the nanoparticles (active ingredient + carrier particle) present in the system, and c_A and c_P are the corresponding concentrations (*e.g.* masses per unit mass or volume of total system). The loading capacity may

vary from around 0 (low) to 100% (high) depending on the nature of the active ingredient and carrier particles, as well as the preparation conditions used to fabricate the nanoparticles.

The *encapsulation efficiency* is defined as the percentage of active ingredient added to a system that is actually incorporated into the carrier particles themselves (rather than in the surrounding fluid):

$$\text{EE} = 100 \times m_{A,E} / m_{A,T} = 100 \times c_{A,E} / c_{A,T} \quad (3)$$

Here $m_{A,E}$ and $m_{A,T}$ are the masses of the active ingredient trapped within the nanoparticles (encapsulated) and the total mass of active ingredient in the system (encapsulated + non-encapsulated), and $c_{A,E}$ and $c_{A,T}$ are the corresponding concentrations. In principle, the encapsulation efficiency can range from 0% (poor) to 100% (good) depending on how well the nanoparticles are able to incorporate the active ingredient relative to the surrounding medium.

The *retention efficiency* is defined as the percentage of active material initially present within the carrier particles that remains inside them after some specified storage period or after exposure to a specific environmental stress:

$$\text{RE} = 100 \times m_{A,t} / m_{A,0} = 100 \times c_{A,t} / c_{A,0} \quad (4)$$

Here, $m_{A,t}$ and $m_{A,0}$ are the masses of the encapsulated active ingredient within the nanoparticles at time t and zero, respectively, and $c_{A,t}$ and $c_{A,0}$ are the corresponding concentrations. The retention efficiency may range from close to 0% (all released) to 100% (all

retained) depending on the system. The amount of an active ingredient that is released from the nanoparticles after a certain time or after exposure to environmental stresses is simply given by: %Released = 100 – RE.

The precise nature of the analytical methods used to measure LC, EE and RE depend on the nature of the active ingredients and carrier matrix within the nanoparticles. To determine these parameters the analytical techniques need to be able to measure the amount of active ingredient inside and/or outside of the nanoparticles, as well as the total amount of active ingredient in the system. In general, the analytical methods available can be classified into two broad groups depending on the nature of sample preparation required to carry out the analysis: non-destructive and destructive.

2.2.2.1. Non-destructive methods

Ideally, an analytical method should be able to measure the concentrations of an active ingredient inside and outside of the nanoparticles *in situ* without destroying the samples being analyzed. The loading, encapsulation, retention and release properties of an active ingredient could then be monitored over time using the same sample without altering its physicochemical or structural properties. Only a few analytical methods are available that are capable of *in situ* and non-destructive analysis of nanoparticle suspensions. Instruments based on nuclear magnetic resonance (NMR) are one of the most powerful methods of measuring the concentrations of substances in different environments within multiphase materials (Heins et al., 2007; Hey and Alsagheer, 1994; Mantle, 2013). Instruments based on electron paramagnetic resonance (EPR) can also be used for this purpose provided a suitable spin-probe is available (Berton-Carabin et al., 2012; Berton-Carabin et al., 2013). Front-face fluorescence methods can be used to provide

information about changes in the local environment of fluorescent ingredients within nanoparticle suspensions without altering sample properties (Castelain and Genot, 1996; Panya et al., 2012).

In situ measurements of the concentration of active ingredients within complex systems can also be made using fiber optic systems based on absorption of UV, visible, or infrared radiation (Guillot et al., 2013; McFearin et al., 2011). A fiber optic probe is dipped into the sample and either the transmission or absorption spectra is measured, which can be used to provide information about the concentration of an active ingredient near the probe. Specialized optical microscopy methods can be used to measure the concentration of a substance as a function of location and time, which enables retention and release kinetics to be monitored, *e.g.*, fluorescence, Raman, or FTIR microscopy (Everall, 2010; Mauricio-Iglesias et al., 2009; Perry et al., 2006).

Ion selective electrodes can be used to non-destructively measure the concentration of specific ions (such as Na^+ and Ca^{2+}) within aqueous solutions (Kotanen et al., 2012; Pflaum et al., 2013), and can therefore be used to monitor the release of these ions from nanoparticles. Similarly, electrical conductivity measurements can be used to measure the release of ions from nanoparticles (Lutz et al., 2009).

2.2.2.2. *Destructive methods*

In many cases, the amount of active ingredient inside and outside of the nanoparticles in a delivery system can only be ascertained after the nanoparticles are separated from the surrounding matrix (see earlier section). The concentration of active ingredient within the nanoparticles and/or the matrix can then be measured using appropriate analytical tools, such as

wet chemistry, chromatography, electrophoresis, or spectroscopy. Numerous approaches are available to isolate nanoparticles from the surrounding matrix, including matrix disruption, selective precipitation, gravitational separation, centrifugation, filtration, and dialysis (Nielsen, 2010; Stockmann and Schwarz, 1999). When nanoparticles are suspended in a fluid phase (such as an aqueous solution or an organic solvent), it is possible to measure the concentration of active ingredient present within the fluid after the nanoparticles have been removed. For example, UV-visible or fluorescence spectrophotometry could be used to detect a substance in solution that absorbs electromagnetic radiation, whereas ion selective electrodes could be used to detect the presence of specific mineral ions (such as sodium or calcium). The concentration of active ingredient encapsulated within the nanoparticles can then be inferred from knowledge of the total amount present and the non-encapsulated amount: $m_{A,E} = m_{A,T} - m_{A,NE}$. The total amount present may be known from a mass balance calculation, or it may be measured on the overall sample. In some cases, additional steps are needed to separate the active ingredient from the fluid phase or total system before it can be analyzed, *e.g.*, concentration, evaporation, or solvent extraction (Nielsen, 2010). Organic solvents are commonly used to extract lipophilic active ingredients from nanoparticle-based delivery systems prior to analysis by chromatographic or spectrophotometric methods. Rotary evaporation can be used to concentrate an active ingredient in a sample prior to analysis by removing some of the surrounding water or organic solvent (Nielsen, 2010).

One simple approach for measuring the amount of non-encapsulated active ingredient present outside of the nanoparticles in a delivery system is dialysis (Baker, 1987; Moreno-Bautista and Tam, 2011). The delivery system is placed within a dialysis bag that has pores

smaller than the nanoparticles, but larger than the active ingredient (**Figure 3**). The concentration of active ingredient present within the liquid surrounding the nanoparticles can then be determined by measuring the amount that passes through the dialysis bag. Specialized measurement cells containing semi-permeable membranes can also be used to carry out this type of experiment (Baker, 1987).

Overall, we recommend that researchers report the loading capacity, encapsulation efficiency, and retention efficiency of any food-grade nanoparticles used as delivery systems for active ingredients. As mentioned earlier, the precise analytical techniques used will depend on the nature of the carrier matrix and active ingredient within the nanoparticles. Researchers should therefore clearly report the analytical instruments and methodology used to measure the LC, EE and RE of their nanoparticles so that others can ascertain the appropriateness of the methods used and duplicate the research if required.

Particle concentration

It is usually important to have knowledge of the total concentration of nanoparticles present in a sample after they have been fabricated. Sometimes the total nanoparticle concentration is known because of the nature of the ingredients and processing operating used to fabricate them, but in other cases it may be necessary to measure the particle concentration, and therefore suitable analytical methods are needed. Before describing these methods, it should be highlighted that particle concentration can be reported in a number of ways (**Table 3**), and it is important to clearly report which method is being used when expressing this parameter.

A number of analytical methods are available to determine the total concentration of particles in a nanoparticle suspension. If there is not an appreciable level of solutes present in

the surrounding liquid, then the nanoparticle suspension can simply be dried and weighed (provided the particle concentration is high enough to give good sensitivity). If there are solutes present, then the nanoparticles may be separated by centrifugation, dialysis, filtration or selective precipitation, and then dried and weighed. If the nanoparticles contain a unique constituent (such as a protein, lipid, or carbohydrate) that can be measured using one of the analytical methods mentioned in the previous section, then the nanoparticle concentration may be determined using one of these methods. If the nanoparticles scatter light appreciably, then their concentration may be determined by simply measuring the turbidity once a suitable calibration curve of turbidity *versus* particle concentration has been established. The main limitation of this method is that the turbidity of a colloidal dispersion depends on the size of the particles, as well as their concentration. This method may therefore be most suitable when the size of the nanoparticles is already known or can be measured independently. In addition, many nanoparticles may only scatter light weakly because of their small size relative to the wavelength of light, and may therefore not give a strong change in turbidity with particle concentration.

Particle size distribution

Nanoparticle size is one of the most important factors influencing their physicochemical properties and functional performance, *e.g.* optical properties, stability, release characteristics, and biological fate (Lesmes and McClements, 2009; McClements et al., 2009b; McClements and Li, 2010b). It is therefore important to be able to reliably measure and report nanoparticle size. Typically, nanoparticle size characteristics are represented by a particle size distribution (PSD) in combination with a measure of the central tendency (such as mean diameter) and width of the distribution (such as the polydispersity index). When reporting particle size data it is always

important to specify whether the particle dimensions are given as radius (r) or diameter (d). It should also be noted that there are many ways of calculating mean particle sizes from particle size distributions, and it is important to specify the actual one used when reporting data, *e.g.*, d_{32} , d_{43} , or d_z (**Table 4**).

In general, there are a number of different analytical instruments that can be used to measure nanoparticle size, including microscopy, light scattering, particle tracking, electrical pulse counting, optical pulse counting, centrifugal methods, field flow fractionation, and ultrasonic spectroscopy (Colfen and Antonietti, 2000; Krpetic et al., 2013; McClements, 2007; Pecora, 2000). Nevertheless, many of these methods require specialized equipment that is not widely available for general application. In this section, we therefore focus on the most widely used analytical methods of measuring particle size: dynamic light scattering (DLS) and static light scattering (SLS). Microscopy methods are also widely used to provide information about particle dimensions, but these are discussed in the following section on measurement of particle morphology and organization.

Static Light Scattering

Static light scattering (SLS) is one of the most widely used methods to determine the size of the particles in colloidal dispersions (McClements, 2007). It is based on the principle that the light scattering pattern (intensity *versus* scattering angle) produced when a monochromatic light beam propagates through a dilute particle suspension depends on the particle size distribution. SLS instruments come with software containing a mathematical model (usually the “Mie theory”) that relates the measured scattering pattern of a particle suspension to the characteristics of the particles it contains (*i.e.*, concentration, diameter, and refractive index). The software

finds the particle size distribution that gives the best-fit between the measured scattering pattern and a theoretically predicted one, and then reports the data as a table or plot of particle concentration *versus* particle size (*e.g.*, **Figure 5**). These mathematical models assume that the particles are homogeneous spheres with well-defined refractive indices. To calculate the PSD the model requires information about the complex refractive index of both the particles and the dispersing medium (usually water).

Commercial SLS instruments are sensitive to particles within the range of about 20 nm to 2000 μm , but can only reliably measure samples with mean diameters somewhat higher than the lower limit (*e.g.*, around 60-80 nm). These instruments normally require that the droplet concentration is relatively low (< 0.1 wt%) so that the laser beam can pass through and to avoid multiple scattering effects (since the mathematical model used assumes single scattering). Some nanoparticle suspensions may therefore need to be diluted considerably prior to analysis, which must be done carefully to avoid altering the microstructure (see later). Another potential disadvantage of conventional SLS instruments is that they are not particularly sensitive to particles with diameters less than about 100 nm, and therefore they may not be able to characterize many types of food-grade nanoparticles.

Dynamic Light Scattering

Dynamic light scattering (DLS) is probably the most appropriate and most widely used particle sizing technology for characterizing the dimensions of nanoparticles (McClements, 2007; Pecora, 2000). Instruments based on this principle measure the random fluctuations in the intensity of light scattered by the particles in a colloidal dispersion when they change their positions due to Brownian motion (**Figure 6**). The frequency of the intensity fluctuations

depends on the rate at which the particles change their positions and therefore on their size: smaller particles move more rapidly than larger ones, and therefore give more rapid intensity fluctuations. A sample is analyzed by recording the intensity fluctuations of the scattered wave at a particular scattering angle, and then using a suitable mathematical model to convert these fluctuations into a particle size distribution (**Figure 6**).

Commercial DLS instruments are sensitive to particles with diameters in the range from about 1 to 3000 nm, and therefore cover the entire range of sizes for nanoparticles. For this reason, they are one of the most appropriate analytical tools for characterization of the dimensions of nanoparticle suspensions, and are therefore widely used by many research laboratories. The prevalence of DLS instruments can be seen in **Table 2**, which shows that most studies on food-grade nanoparticles use this method for particle size determination. It is important that the particle concentration of the nanoparticle suspension being analyzed falls within an appropriate range, which depends on the design of the instrument being used. Some DLS instruments utilize the light waves that have been transmitted through a suspension to measure the intensity fluctuations, and are therefore only suitable for analysis of dilute systems (< 0.1 wt%). Other instruments utilize the back-scattered light and are therefore suitable for characterizing both dilute and concentrated nanoparticle suspensions (0.001 to 10 wt%).

Practical Considerations

Commercial SLS and DLS instruments are simple to operate and maintain, and provide full particle size distributions within a few minutes, which has led to their widespread use for characterizing the dimensions of food-grade nanoparticles. However, to obtain reliable information it is important to understand the underlying physical principles of the instrument, to

operate and maintain it properly, and to be aware of and eliminate any potential sources of measurement errors (McClements, 2005). These errors may come from a variety of sources: sample preparation (*e.g.*, dilution or stirring) alters the structure of the material being analyzed; the instrument is not properly cleaned or operated; an inappropriate mathematical model is used to convert the raw data into a PSD; the particles do not conform to the assumptions made in the mathematical model (*e.g.*, they are non-spherical or inhomogeneous); incorrect physical parameters are used in the mathematical model (*e.g.*, refractive index or viscosity); the particle size or concentration are not in the appropriate range for the instrument used.

Many commercial light scattering instruments require some form of sample preparation prior to carrying out the measurements, *e.g.*, stirring to ensure homogeneity, dilution to prevent multiple scattering, or temperature control. These procedures may alter the properties of the nanoparticles being analyzed so that the measured PSD is different from that in the original sample. For example, dilution and stirring may cause appreciable alterations in the particle size distribution, particularly in suspensions containing weakly flocculated particles or those that are sensitive to aggregation (McClements, 2007). When dilution is required, it is usually important to carry it out using a solution that has the same properties as the continuous phase of the original sample, *e.g.*, pH, ionic strength, and temperature. In addition, it is always important to report the values of these parameters when presenting particle size data, since they often have a major impact on nanoparticle stability.

Most commercial SLS and DLS instruments use some form of mathematical model to convert the measured light scattering data into a PSD. These models are usually based on some simplifying assumptions, such as the particles are spherical, homogeneous, and non-interacting.

To obtain reliable information about the PSD it is therefore important to ensure that the properties of the sample being analyzed conform to the assumptions used in the mathematical model. Many food-grade nanoparticles are not simple isolated homogeneous spheres, which means that the data obtained from a light scattering instrument should be treated with some caution. If the nanoparticles are flocculated, then the mathematical models used are unlikely to work correctly, because the scatterers are non-spherical, inhomogeneous, and interacting. Consequently, the results obtained may only provide a qualitative indication of the actual particle size distribution, or may even be completely unreliable.

It is important to use any commercial particle size analyzer correctly by carefully following the instructions provided by the instrument manufacturer. In particular, it is usually important to thoroughly clean particle sizing instruments regularly to ensure that they work correctly, and to calibrate them periodically with standards (*i.e.*, particles with known sizes). In addition, it is important to ensure that the nanoparticles analyzed fall within the particle size and concentration ranges that the instrument is sensitive to. An SLS instrument will not reliably detect very fine particles present within a sample (*e.g.*, $d < 50$ nm), whereas a DLS instrument will not reliably detect any large particles or aggregates (*e.g.*, $d > 5000$ nm). If the nanoparticle suspension being analyzed has a significant fraction of particles out of the range of detection of the instrument, then misleading results will be obtained.

For these reasons, it is usually good practice to always confirm particle size data obtained by light scattering (or other particle sizing methods) using some form of microscopy.

Particle morphology and organization

Food-grade nanoparticles may have a variety of different structures (McClements, 2014).

Many nanoparticles are roughly spherical in shape, but they may also have other shapes, such as needles, ellipsoids, or cuboids (**Figure 2**). Nanoparticles may also have complex internal structures, such as homogeneous, core-shell, or dispersion. They may also be organized into different types of structural arrangements, such as isolated particles, chains, or flocs. Nanoparticle morphology and organization plays an important role in determining their physicochemical and functional properties, and therefore it is important to have analytical methods to measure these properties.

Various microscopy methods are available for characterizing the morphology or organization of nanoparticles, with the most common being optical, electron, and atomic force microscopy (Morris et al., 1999; Murphy, 2012). Each of these methods works on different physical principles, and has advantages and limitations for particular applications (Russ, 2011). Nevertheless, any microscopy method must have three main attributes: resolution; magnification; and, contrast (Aguilera et al., 1999). *Contrast* is the ability to distinguish the objects in an image from the background and from other objects. *Resolution* is the ability to distinguish two different objects that are in close proximity. *Magnification* is the number of times the image appears larger than the actual object being observed. Modern microscopes are typically attached to computers that capture and store a digital image of the sample being observed, which can then be analyzed using digital processing software to obtain information about morphology and organization, *e.g.*, particle size distribution, particle shape, internal structure, aggregation state, location, and/or chemical composition (Russ, 2004, 2011).

Optical microscopy

An optical microscope uses electromagnetic radiation in the visible region to provide images

of a sample (Mertz, 2009). An optical microscope typically consists of a light source, a series of lenses, and an eye piece or digital camera. The lenses direct the light waves through the sample and magnify the resulting image so that it can be observed or recorded. The *resolution* of an optical microscope is determined by the wavelength of light used and the mechanical design of the instrument. In theory, the resolution of an optical microscope is around 200 nm, but in practice it is difficult to obtain reliable measurements below about 1000 nm due to the mechanical limitations of the microscope components and the Brownian motion of small particles. Optical microscopes therefore have very limited application for the characterization of the dimensions or morphology of individual nanoparticles because these particles are too small to observe directly, but they may be useful for characterizing the overall structure of flocculated nanoparticles or for determining the location of nanoparticles in complex systems.

The image quality of optical microscopes can be improved using specialized devices that enhance the contrast between different components, such as *phase contrast* or *differential interference contrast (DIC)* microscopy (Murphy, 2012). Optically anisotropic nanoparticles, such as fat crystals, liquid crystals, or native starch granules, can be distinguished from an isotropic background using *polarization* light microscopy (Aguilera et al., 1999). The aggregation state or location of nanoparticles can be determined using stains or dyes that specifically bind to a component of interest (*e.g.* protein, polysaccharide, or lipid) or that preferentially partition into a particular phase (*e.g.*, oil or water). Further improvements in image quality can be achieved using laser scanning confocal microscopy (LSCM). LSCM allows one to obtain high quality two-dimensional images of a sample in a particular plane, or to construct three-dimensional images by taking a series of two-dimensional slices in the vertical direction

(Pawley, 2006; Plucknett et al., 2001). LSCM is particularly useful for examining the aggregation state, location, and movement of specific components within multicomponent systems because of the possibility of using fluorescent probes to tag specific molecules or phases (Loren et al., 2007).

Other advanced forms of optical microscopy have been developed that can provide images of samples based on differences in their chemical composition, such as Fourier Transform infrared (FTIR) imaging, surface enhanced resonance spectroscopy (SERS) imaging, and X-ray microscopy (Byelov et al., 2013; Cialla et al., 2012; Hertz et al., 2012; Levin and Bhargava, 2005; Stewart et al., 2012). These methods produce images that highlight specific components (such as protein, lipids or carbohydrates) within samples, and may therefore be used to provide information about the aggregation state, location or movement of nanoparticles within materials.

Electron microscopy

Electron microscopy is often the most appropriate tool for characterizing the morphology and organization of nanoparticles due to its ability to detect structural elements that are too small to see using optical microscopes, *i.e.* $d < 1000$ nm (Klang et al., 2012; Klang et al., 2013). The most powerful electron microscopes are sophisticated pieces of equipment that are relatively expensive to purchase and maintain, and are therefore only available at large research institutions. Nevertheless, bench-top versions are becoming more widely available that have less magnification and resolution, but that are cheaper and easier to use, and therefore more suitable for routine analysis.

Electron microscopes use electron beams to provide detailed information about the microstructure of materials. A major advantage of using electron beams (rather than light

waves) is that they have much smaller wavelengths and so can be used to examine much finer structures. In principle, the *resolution* of an electron beam is around 0.2 nm, but in practice it is usually around 1 nm, even with the most powerful instruments, due to mechanical limitations of magnetic lenses. It should be noted that the resolution may be much worse than this value for bench-top instruments. The *contrast* in the images obtained by electron microscopy is primarily due to differences in the electron density of the different components in a sample, and can often be improved by using stains that specifically bind to a particular component. The *magnification* depends on the type of instrument used, and can be as high as several million times for the most powerful instruments, which enables very small (\approx nm) structures to be observed.

Two types of electron microscope are commonly used to examine the morphology and organization of nanoparticles: Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) (Egerton, 2008; Murphy, 2012). Conventional TEM and SEM instruments require that the samples being analyzed are placed in a high vacuum chamber so that the electron beam is not scattered by molecular species in gases, which would lead to a deterioration in image quality. Samples therefore had to undergo extensive preparation (*e.g.*, fixation, dehydration) prior to analysis to ensure that they were free of volatile constituents that might evaporate and reduce image quality, such as water or volatile organic molecules. These types of sample preparation procedures can lead to artefacts in the final images for certain samples. More recently, environmental SEM and TEM instruments have become available that are capable of obtaining images of materials in a gaseous environment, and so there are far fewer problems with artifact generation during sample preparation (Donald, 1998; Donald et al., 2000; Jinschek, 2013; Jinschek, 2014).

2.5.2.1. *Transmission Electron Microscopy*

TEM is the most suitable method for providing detailed information about very fine structures in materials, *e.g.*, nanoparticles or thin interfaces (Danino, 2012; Dudkiewicz et al., 2011; Klang et al., 2013). In this case, an electron beam is generated, focused, and then passed through a sample using a series of magnetic lenses (**Figure 7**). Part of the electron beam is absorbed or scattered by the sample, but the rest is transmitted. The transmitted part is magnified and projected onto a screen to create an image of the sample (**Figure 7**). The fraction of electrons transmitted by a substance depends on its electron density: the higher the electron density, the lower the fraction of electrons transmitted and the lighter the image. Constituents with different electron densities therefore appear as regions of different intensity on the final image. The magnification factor for TEM is typically in the range of about 10^2 to 10^6 times, which enables very fine structures to be observed.

Electrons are highly attenuated by most materials and therefore the samples to be analyzed must be extremely thin to allow sufficient electrons through to be detected. The samples analyzed by TEM are therefore usually much thinner ($\sim 50 - 100$ nm) than those analyzed by optical microscopy (~ 1 μm to a few mm). The electron density contrast between the major constituents in suspensions of food-grade nanoparticles is often relatively small, and so it is difficult to distinguish them. For this reason, the density contrast is usually enhanced by selectively staining specific components within the sample using heavy metal salts that have high electron densities, such as lead, tungsten, or uranium. These salts selectively bind to specific components thereby enabling them to be distinguished from the rest of the sample. The need to utilize very thin dehydrated specimens that often require staining means that sample preparation

is more time-consuming and laborious for TEM than for other forms of microscopy, and may lead to appreciable artifacts in the images obtained. On the other hand, the level of detail that can be observed cannot be obtained by other forms of microscopy. A representative TEM image of gold nanoparticles absorbed by model epithelium cells measured in our laboratory is shown in **Figure 7**.

2.5.2.2. Scanning Electron Microscopy

SEM is commonly used to provide images of the surface topography of specimens (Egerton, 2008), and can be used to provide useful information about the morphology and organization of food-grade nanoparticles (Hosseini et al., 2013; Paques et al., 2014). It produces images based on detection of secondary electrons generated by a sample when it is bombarded by an electron beam, rather than on detection of the electrons that have passed through the specimen (as in TEM). A focused electron beam is directed at a particular point on the surface of the sample being analyzed. Some of the energy associated with the electron beam is absorbed by the material causing it to generate secondary electrons that leave the surface of the sample and are captured by a detector. An image of the specimen is obtained by scanning the electron beam in an x-y direction over its surface and recording the intensity of the electrons generated at each location using a suitable detector. The resulting SEM image has a three-dimensional appearance (**Figure 8**), in contrast to the two-dimensional appearance of TEM images (**Figure 7**).

Sample preparation for SEM is considerably easier and tends to produce fewer artifacts than TEM (Egerton, 2008). Because an image is produced by secondary electrons generated at the surface of a specimen, rather than by an electron beam that passes through a specimen, it is not necessary to use very thin samples. Even so, the specimens used in conventional SEM often

have to be cut, fractured, fixed, dehydrated, and sputter coated, which may alter their structures. Nevertheless, many of these problems have been overcome with the introduction of environmental SEM as mentioned earlier (Donald, 1998; Donald et al., 2000). The resolving power of a powerful analytical SEM instrument is around 3 to 4 nm (but it is considerably higher for cheaper bench-top instruments), which is an order of magnitude worse than TEM, but about two or three orders of magnitude better than optical microscopy. Another major advantage of SEM over optical microscopy is the large *depth of field*, which means that images of relatively large structures are all in-focus. An image of the lipid nanoparticles in hydrocarbon oil-in-water nanoemulsions acquired using SEM is shown in **Figure 8**.

Atomic force microscopy

Atomic force microscopy (AFM) can provide information about the structure of materials at the molecular and colloidal level and is therefore particularly suitable for analyzing food-grade nanoparticles (Morris et al., 1999; Sitterberg et al., 2010). An atomic force microscope scans a tiny probe across the surface of the sample being analyzed, and generates a topological image by analyzing the interaction between the probe and sample surface using a suitable detection device (**Figure 9**). The resolution of AFM depends on the size and shape of the probe, as well as how accurately it can be positioned relative to the sample surface. AFM instruments typically enable lateral resolution of structures on the order of nanometers and vertical resolutions on the order of tenths of nanometers. AFM can be used to analyze both wet and dry samples, which makes it suitable for application to many different kinds of samples, with relatively little sample preparation compared to electron microscopy. Nevertheless, the images obtained using AFM are often not as informative as those obtained using TEM and SEM, and sample preparation still has

to be carried out carefully to avoid artefacts.

Practical Considerations

To generate accurate and meaningful results it is essential that microscopy instruments are used correctly (Aguilera et al., 1999). Samples have to be carefully prepared to avoid introducing artifacts into the images that are unrepresentative of the structures being analyzed. Image acquisition and analysis should also be carried out carefully to avoid introducing artifacts, and to ensure high quality representative images. Different microscopy methods have advantages and disadvantages for particular applications. Optical microscopy is the simplest and cheapest method to use, but it is only suitable for providing detailed information about the properties of relatively large structures (> 1000 nm). For this reason, it is normally only used to provide information about the location, movement, or aggregation of nanoparticles rather about the structure of individual nanoparticles. A major advantage of optical microscopy methods is the possibility of differentiating the location of different constituents within a system (e.g., fats, proteins, and carbohydrates) using stains, dyes, or polarization. In addition, specialist measurement cells can be purchased that can be useful for characterizing nanoparticle systems, such as variable temperature, environmental control, micromanipulation, or shear cells (van der Linden et al., 2003).

In many applications, electron microscopy is the most appropriate technique for characterizing the properties of food-grade nanoparticles since it can be used to detect structures in the 1 to 1000 nm range. The major disadvantage of electron microscopy is that extensive sample preparation is required, which is often time consuming, labor intensive, and may introduce artifacts into the images (Aguilera et al., 1999). Atomic force microscopy is also used

for nanoparticle characterization (Sitterberg et al., 2010), but it is less versatile than electron microscopy and often produces images that are more difficult to interpret. In summary, we strongly recommend that researchers characterize the food-grade nanoparticles they have fabricated using electron microscopy (SEM or TEM) when reporting their results.

Particle charge

The electrical charge on food-grade nanoparticles is important because it determines how they interact with other charged species in their environment, such as other molecules, particles, or surfaces. The origin of the charge on nanoparticles can be attributed to the presence of ionized constituents at their surfaces, such as ionic surfactants, phospholipids, proteins, polysaccharides, and mineral ions (McClements, 2007). The electrical characteristics of nanoparticles depend on the type, concentration, and location of any ionized groups at their surfaces, as well as the ionic composition and physical properties of the surrounding liquid.

The electrical characteristics of nanoparticles are characterized by their surface charge density, surface electrical potential, and ζ -potential (Hunter, 1986; McClements, 2005). The surface charge density is the number of electrical charges per unit surface area. The surface electrical potential is the free energy required to increase the surface charge density from zero to a finite value, by bringing charges from an infinite distance to the surface through the surrounding medium. It therefore depends on the surface charge density, as well as the ionic composition and dielectric constant of the surrounding medium due to electrostatic screening effects. The ζ -potential is the electrical potential at the "shear plane", which is defined as the distance away from the particle surface below which any counter-ions remain strongly attached to the particle when it moves in an electrical field. Practically, the ζ -potential is the most widely

used parameter for representing the electrical characteristics of nanoparticles because it is much easier to measure than the surface charge density or electrical potential. In addition, it inherently takes into account the fact that the electrical properties of the particle may be altered by other ionic species adsorbed to its surface. It is highly recommended that the electrical properties of food-grade nanoparticles are characterized by measuring the ζ -potential *versus* pH profile under solution conditions that mimic the final application (*e.g.*, ionic composition and temperature). A variety of analytical instruments are commercially available that can be used to measure the electrical charge (ζ -potential) on nanoparticles, with the two most important being particle micro-electrophoresis and electro-acoustics.

Micro-electrophoresis

Commercial instruments based on particle micro-electrophoresis are by far the most commonly used to characterize the electrical properties of nanoparticles. These instruments measure the velocity and direction that charged nanoparticles move in a well-defined applied electrical field (Delgado et al., 2007; McClements, 2007). A nanoparticle suspension is placed into a measurement cell that contains a pair of electrodes, and then a well-defined electrical field is applied (**Figure 10**). The electric field causes the charged particles to move towards the oppositely charged electrode at a speed that depends on the magnitude of their charge and the viscosity of the surrounding liquid. The instrument calculates the electrophoretic mobility from measurements of particle velocity, and then uses a mathematical model in the instrument software to convert this information into a ζ -potential. The movement of the particle in a measurement cell can be followed using various techniques, with the most common being laser light scattering. Most commercial ζ -potential instruments based on light scattering require that

the nanoparticle concentration be relatively low (< 0.1 wt%) so that light can be transmitted through the system and multiple scattering effects avoided. Consequently, many nanoparticle suspensions have to be diluted prior to analysis, which must be done carefully to avoid changes in the electrical characteristics of the nanoparticles (see below).

Electro-Acoustics

Electro-acoustic instruments are also commercially available that can measure the ζ -potential of nanoparticles in suspensions, but they are much less commonly used than micro-electrophoresis methods (Dukhin and Goetz, 2001; McClements, 2007). These instruments work by applying an oscillating electric field to a sample and measuring the resulting acoustic signal generated by the oscillating particles, or by applying an oscillating acoustic field and measuring the resulting electric signal generated by the oscillating particles. The resulting signals can be converted to the ζ -potential of the nanoparticles using a suitable mathematical model. The major advantage of electro-acoustic techniques is that they can be applied to concentrated nanoparticle suspensions ($\phi \leq 50\%$) without the need for sample dilution. Nevertheless, particle-particle interactions at higher concentrations may make interpretation of the measurements unreliable.

Practical Considerations

To obtain reliable particle charge measurements it is important to operate ζ -potential instruments properly and to be aware of any potential sources of error (Delgado et al., 2007; McClements, 2005, 2007). Errors may come from a variety of sources, including the sample itself, the sample preparation procedure, the mechanical operation of the instrument, and the mathematical model used to convert the measured signal into a particle charge. The dilution of a nanoparticle suspension may be a major source of error in ζ -potential measurements made using

micro-electrophoresis instruments. Dilution can cause a significant alteration in the ζ -potential of the nanoparticles due to the potential change in pH, ionic strength, and/or composition of the surrounding solution. In particular, dilution may cause some of the material that was originally adsorbed to the nanoparticle surfaces to move off them. Ideally, one should dilute a nanoparticle suspension using a medium that has the same composition as the solution surrounding the particles in the original sample. This can be achieved by preparing a solution with the same composition as that surrounding the nanoparticles, or by centrifuging a nanoparticle suspension and collecting the serum phase. At the very least, one should ensure that the pH and ionic strength of the diluting solution is similar to that of the original nanoparticle suspension. However, even if the pH and ionic strength are kept the same, there is still the possibility that dilution will change the partitioning of a charged substance between the nanoparticle surface and the surrounding liquid, thereby altering the ζ -potential, *e.g.*, an adsorbed polyelectrolyte, surfactant, or mineral ion may be partially displaced after dilution. In summary, we strongly recommend that researchers use micro-electrophoresis to characterize the electrical properties of nanoparticles (preferably as a function of pH), and that sample preparation, measurement and analysis are carried out carefully to avoid introducing errors. In addition, it is always important to report the pH, ionic strength and temperature at which the reported data were measured.

Particle physical state

Food-grade nanoparticles may be solid, semi-solid, or liquid depending on their composition, environmental conditions, and thermal history (McClements, 2014). The physical state of the nanoparticles is often important because it determines their stability (*e.g.*, to gravitational separation, aggregation, or Ostwald ripening) and their functional performance

(*e.g.*, protection, retention, and release). It is therefore important to have analytical tools that can provide information about the physical state of food-grade nanoparticles. In this section, a brief overview of some of the major analytical tools that can be used to measure the physical state of nanoparticles is given.

Dilatometry

This method is based on measuring changes in the overall density of a nanoparticle suspension. The density of a material usually changes appreciably in response to a change in its physical state *e.g.*, crystallization or melting (McClements, 2007). For example, the density of solid lipids is usually much higher than that of liquid lipids. For fluid nanoparticle suspensions, this method is relatively simple, rapid, and inexpensive to perform using widely available instruments, such as digital densitometers or density bottles. Typically, the overall density is measured as a function of either temperature or time to monitor phase transitions.

Differential scanning calorimetry

Different scanning calorimetry (DSC) is one of the most commonly used analytical tools for providing information about the physical state of nanoparticles. It is based on measurements of the enthalpy changes (heat released or absorbed) when a sample is subjected to a controlled temperature scan *i.e.*, cooling or heating (Qian et al., 2012; Shrestha et al., 2007; Shukat et al., 2012; Silalai and Roos, 2010). DSC can be used to provide valuable information about the total amount of material that undergoes a phase transition, the polymorphic form of a material, and the location of transition temperatures (such as melting or crystallization points).

Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is based on measurements of the response of a sample

to an applied pulse of radio-frequency electromagnetic radiation (Gladden, 1995; McClements, 2005). The hydrogen nuclei within a sample move to an excited state after application of the pulse, but then decay back to their unexcited states. The decay rate can be measured using magnetic coils and depends on the physical state of the substance: the signal from solids decays much more rapidly than that from liquids. Thus, the physical state of a sample can be determined by measuring the decay rate of the NMR signal. This method can be used to monitor the total solid content of nanoparticles, and to establish phase transition temperatures such as melting and crystallization points.

Ultrasonics

This method is based on precise measurements of the ultrasonic velocity and/or attenuation coefficient of a nanoparticle suspension, which may change appreciably when a nanoparticle undergoes a phase transition (Awad et al., 2012; McClements, 1997). This method may be used to non-destructively monitor phase transitions such as melting, crystallization, polymorphism, and glass-rubbery transitions. However, this method is not widely available and therefore only tends to be used in a few specialist laboratories.

Scattering and Spectrometry Methods

X-ray diffraction is one of the most powerful tools for measuring the spatial arrangement of molecules within a material, and can therefore provide valuable information about their physical state (liquid, crystalline, or amorphous) and polymorphic form (Hartel, 2013). This method is particularly powerful when the measurements are carried out as a function of temperature or time, since then information about the nature and kinetics of phase transitions can be obtained (Bunjes, 2011; Gupta and Rousseau, 2012; Relkin et al., 2011). Raman spectroscopy can also

provide information about the physical state and crystal form based on analysis of changes in the electromagnetic spectrum when a material undergoes a state change (Bresson et al., 2011; Celedon and Aguilera, 2002).

Microscopy

Various forms of microscopy can be used to provide information about the physical state of nanoparticles. Optical microscopy combined with crossed-polarizers can be used to determine the presence and location of crystalline or liquid crystal regions in a sample (Li et al., 2012b; Li et al., 2012c). TEM can be used to provide detailed information about molecular packing within nanoparticles, such as triglyceride molecules in solid lipid nanoparticles (Bunjes et al., 2007; Kuntsche et al., 2011).

Practical Considerations

There are a number of practical considerations that must be taken into account when identifying a suitable analytical tool to provide information about the physical state of food-grade nanoparticles. First, it is important that the analytical instrument is sensitive to the particular kind of phase transition occurring within that sample, *e.g.*, solid-liquid, polymorphic form, sol-gel, or glass-rubbery. Second, the instrument should be capable of covering the temperature range over which this transition occurs. Third, it is important that any sample preparation procedure does not alter the physical state of the nanoparticles being analyzed, *e.g.*, by causing melting, crystallization, or polymorphic changes. Fourth, in multicomponent systems it may be important to distinguish different kinds of phase transitions associated with different constituents.

General Recommendations for Nanoparticle Characterization

Based on the discussion in the previous sections, we highly recommend that the following analytical tools be used to characterize the properties of food-grade nanoparticles:

- *Particle size distribution* – Dynamic light scattering is the most appropriate method for most applications because it covers the entire range of dimensions for nanoparticles (1 to 1000 nm). However, SLS may be more appropriate if one is characterizing nanoparticle aggregation, since the aggregates formed may be too large to analyze using DLS.
- *Particle charge* – Micro-electrophoresis is the most suitable method for measuring the electrical characteristics of nanoparticles, but sample preparation must be carried out carefully to avoid introducing artefacts.
- *Particle morphology and location*: Transmission electron microscopy is the most useful method for characterizing the morphology and location of nanoparticles because of its high resolution.

In addition, it may be useful to provide information about particle composition and physical state using one or more of the methods mentioned earlier, if this information is unknown. Finally, it is always important to report the pH, ionic composition, and temperature of the medium in which the nanoparticles are suspended, since these parameters often have a major impact on nanoparticle properties.

CHARACTERIZATION OF NANOPARTICLE STABILITY TO ENVIRONMENTAL STRESSES

After fabrication, food-grade nanoparticles may be incorporated into a variety of products that have different compositions and that experience different environmental conditions. In addition, they may experience substantial changes in their environments as they pass through the human gastrointestinal tract (GIT) after ingestion. The physicochemical properties and stability of nanoparticles may change appreciably in response to these alterations in their environments. For this reason, it is important to have knowledge about how nanoparticles behave under a range of environmental conditions. In this section, we initially describe some environmental stress tests that can be used to establish the range of conditions where food-grade nanoparticles are stable, and then we highlight the need to carry out tests under conditions that simulate the actual conditions that nanoparticles experience in the products where they are going to be applied.

General Environmental Stress Tests

Food-grade nanoparticles often need to function over a range of different environmental conditions, which are highly dependent on the final application (McClements, 2007). For example, there may be appreciable variations in pH, ionic strength, solution composition, temperature, and mechanical stresses depending on the application. It is therefore useful to establish the range of environmental conditions where a particular type of nanoparticle can successfully operate. In this section we propose a series of environmental stress tests that can be used to establish the application space of food-grade nanoparticles. Typically, a suspension of nanoparticles would be prepared, subjected to one or more of these stress tests, and then their

stability and properties would be tested using one or more of the methods described previously, *e.g.*, particle size, charge, and aggregation state.

- *Extended storage.* Many commercial products must remain stable for relatively long periods before they are utilized, *e.g.*, weeks, months, or years (McClements, 2007). The resistance of nanoparticles to changes in their properties during long-term storage can be assessed by keeping them in a temperature-, humidity- and light-controlled environment for a fixed period whilst periodically testing their properties. The precise conditions used will depend on the expected shelf-life and storage conditions of the final product. However, a testing protocol that can be used for a wide range of applications is to store samples at temperatures that mimic refrigeration and ambient conditions for an extended period *e.g.*, 6 months at 4, 20 and 37 °C in a dark room to mimic refrigerator, mild climate, and hot climate conditions. Samples can be withdrawn every one or two weeks for analysis, depending on the rate of change in the product.
- *pH and Minerals.* Commercial products may vary considerably in the pH and mineral composition of the aqueous phase, which may have a pronounced impact on the stability and properties of nanoparticles (Lee et al., 2011; Pan et al., 2014). The resistance of products to these effects can be established by preparing them, then dispersing them in aqueous solutions with different pH values (*e.g.*, pH 3 - 8) and mineral concentrations (*e.g.*, 0 - 500 mM NaCl and 0 - 100 mM CaCl₂), and then

testing their properties after they have been stored for a fixed period (*e.g.*, 24 hours or 1 week at 25 °C or ambient temperature).

- *Thermal Processing.* Many commercial products that could contain nanoparticles are subjected to thermal processing during their manufacture or utilization, *e.g.*, pasteurization, sterilization, or cooking (Lee et al., 2011; Rao and McClements, 2011). In addition, a product may experience considerable temperature fluctuations during storage and transport. The stability of nanoparticle suspensions to heating can be tested by placing them in a temperature-controlled water bath at different temperatures (*e.g.*, 30 to 100 °C for 30 minutes). Alternatively, one can simulate a specific time-temperature profile that a commercial product may actually experience, such as sterilization (*e.g.*, 30 min at 110°C) or pasteurization (*e.g.*, 72 °C for 16 s). The products can then be cooled to ambient temperature and tested.
- *Freeze-Thaw Cycling.* Certain types of commercial products that may contain nanoparticles are subjected to freezing and thawing during their lifetime, such as frozen foods and pharmaceutical preparations (Degner et al., 2014; Donsi et al., 2011; Miao et al., 2014). The resistance of nanoparticle suspensions to freeze-thaw cycling can be established by placing a sample in a freezer at a fixed temperature for a specified time (*e.g.*, -20 °C for 22 hours), then thawing it at ambient temperature (*e.g.*, +20 °C for 2 hours) prior to testing. This procedure can be repeated a number of times to establish the stability to freeze-thaw cycling, with the sample being tested after each cycle.
- *Dehydration.* Some commercial products are dehydrated during the manufacturing process to increase their shelf-life, facilitate transport, reduce storage costs, and

improve convenience *e.g.*, powdered food or pharmaceutical products (Ezhilarasi et al., 2013; Maher et al., 2014). The resistance of nanoparticle suspensions to dehydration can be assessed by drying them, storing them for a fixed period under standardized conditions (*e.g.*, light exposure, oxygen level, relative humidity, and temperature), and then testing them after they have been rehydrated in an appropriate solvent. The most commonly used commercial dehydration method is spray drying, but other methods may be more convenient for certain applications, such as freeze drying when the amount of sample present is limited or when a spray drier is not available.

- *Mechanical stresses.* Many commercial products containing nanoparticles must remain stable when they are subjected to mechanical stresses, such as flow through a pipe, mixing, stirring, or vibration during transport (Mason et al., 2006). The resistance of products to mechanical stresses can be determined by placing them within containers and then subjecting them to a well-defined force for a specified period of time. For example, fluid products may be placed in sealed flasks or beakers and then swirled at a fixed rotational speed in a temperature controlled shaker, *e.g.*, 120 revs/min at 25 °C for 1 week. Alternatively, samples may be placed in a high shear mixer and sheared at a fixed speed for a certain time, *e.g.*, 1000 revs/min at 25 °C for 2 minute. The approach used will depend on the type of mechanical forces that one is trying to simulate. After the mechanical stresses have been applied, the sample can be analyzed to determine any changes in its physicochemical properties or stability.
- *Light Exposure.* Many commercial products contain components that may undergo degradation when exposed to ultraviolet-visible light during their production, storage,

transport and utilization. For example, ω -3 oils, carotenoids, and resveratrol chemically degrade when exposed to UV-visible light for prolonged periods (Allan et al., 2009; Boon et al., 2010; McClements and Decker, 2000). The resistance of samples to this kind of degradation can be established by placing them within controlled light environments that expose them to electromagnetic radiation with a well-defined wavelength, intensity, and duration. Any physical and chemical changes in the material can then be established using the analytical tools discussed earlier.

Suggested environmental stress tests for establishing the stability range of food-grade nanoparticles are summarized in **Table 5**. We recommend that researchers should use a table like this to record the mean particle diameter, electrical charge, and aggregation stability of the nanoparticles they have developed. This would allow for more systematic characterization of the advantages and disadvantages of different kinds of nanoparticles, and would enable product developers to select the most appropriate nanoparticles for a particular application in a more rational manner. In some cases, it may be more appropriate to represent the data as figures rather than as tables, *e.g.*, if particle size or charge is measured over a wide range of pH, ionic strength, or temperature values.

Product-Specific Environmental Stress Tests

If nanoparticles are going to be incorporated into a particular commercial product, then it is important to be sure that they are capable of functioning properly over the specific range of environmental stresses that they will encounter in that specific product. In addition, there may be additional environmental stress tests that are important for some products, *e.g.*, aeration,

chilling, specific thermal processing requirements, sonication, or high pressure treatments. In this case, one would want to develop a series of environmental stresses that closely modeled those that the nanoparticles would actually experience in practice.

Case Study: Stability of Protein-coated Lipid Nanoparticles

In this section, previous experiments on protein-coated lipid nanoparticles from our laboratory are used to demonstrate the applicability of the standardized nanoparticle characterization scheme (Ozturk *et al.* 2014). The influence of a number of environmental stresses on the properties of β -lactoglobulin-coated lipid nanoparticles dispersed in water are shown in **Table 6**. This type of food-grade nanoparticle is positively charged at low pH but negatively charged at high pH, with a point of zero charge around pH 5. The nanoparticles are unstable to aggregation under certain environmental conditions: *pH* – they flocculate around the point of zero charge (pH 5); *Ionic strength* – they weakly flocculate at NaCl concentrations exceeding 200 mM; *Thermal processing* – they strongly flocculate when heated above 60 °C in the presence of salt (150 mM NaCl). The origin of these effects can be attributed to changes in the electrostatic and hydrophobic interactions in the nanoemulsions when environmental conditions are changed. A transmission electron microscopy image and a particle size distribution (dynamic light scattering) of β -lactoglobulin-coated lipid nanoparticles is shown in **Figure 11**. It should be noted that these nanoparticles actually had a different lipid composition (corn oil) than the ones reported in **Table 6** (vitamin E and lemon oil), but the preparation method was similar (high pressure homogenization), which would be expected to result in particles with similar morphologies. In practice, one would report an electron microscopy image of the actual samples being analyzed.

CHARACTERIZATION OF NANOPARTICLE GASTROINTESTINAL FATE

One of the most important applications of food-grade nanoparticles is as oral delivery systems for nutraceuticals and pharmaceuticals (Acosta, 2009; McClements, 2013; Velikov and Pelan, 2008). For these applications, it is important to understand the behavior and fate of the nanoparticles as they pass through the human gastrointestinal tract. In addition, there has been increasing concern about the potential adverse effects of food-grade nanoparticles on human health (Bouwmeester et al., 2009; McClements, 2013; Yada et al., 2014). Assessment of the potential toxicity of nanoparticles within the human body also depends on understanding their gastrointestinal fate after ingestion. A number of analytical approaches have therefore been developed to establish the potential gastrointestinal fate of nanoparticles (Dahan and Hoffman, 2008; Li et al., 2011; McClements and Li, 2010a; Szalai et al., 2014; Williams et al., 2012). Relatively simple *in vitro* chemical methods are typically used in the initial stages of development to rapidly screen different formulations, then animal studies are used to test their *in vivo* efficacy and safety, and finally human feeding studies are used to establish their bioactivity. We recommend that any newly developed food-grade nanoparticle delivery system be passed through a series of *in vitro* and *in vivo* tests to establish its potential biological fate. At a minimum, a standardized *in vitro* method should be used to test the behavior of nanoparticles under conditions that simulate the mouth, stomach, and small intestine (see later).

***In Vitro* Approaches**

In vitro approaches are most suitable for rapidly screening different formulations during the early development of food-grade nanoparticles, as well as for identifying the key physicochemical attributes of nanoparticles that influence their biological fate *e.g.*, composition,

size, charge, and aggregation state (Minekus et al., 2014). A number of *in vitro* approaches have been developed that are suitable for studying the gastrointestinal fate of nanoparticles, which vary in their simplicity, cost, time, reproducibility, and ability to reliably simulate actual GIT conditions. Some of these *in vitro* approaches focus on a specific region of the GIT (such as the mouth, stomach, small intestine, or colon), whereas others utilize a series of sequential steps to more accurately mimic the entire GIT (Li et al., 2011; Minekus et al., 2014; Yoo and Chen, 2006a). Typically, a sample containing nanoparticles is prepared and then subjected to one or more treatments designed to simulate the human GIT. These treatments usually involve mixing the sample with simulated gastrointestinal fluids with specific compositions (*e.g.*, pH, minerals, acids, bases, bile salts, enzymes, *etc.*) under flow conditions and incubation times that mimic particular regions of the GIT. Nanoparticle properties are typically altered when they are exposed to different GIT environments, which may alter their subsequent gastrointestinal fate (*e.g.*, digestion and absorption). In addition, any encapsulated ingredients within the nanoparticles may be retained, released, or chemically modified. It is therefore important to have analytical tools and experimental protocols to measure both the fate of the nanoparticles themselves and of any active ingredients trapped within them. In this section, we highlight a number of methods suitable for characterizing the potential gastrointestinal fate of food-grade nanoparticles, and then propose a minimum set of measurements that should be carried out.

Passage through the gastrointestinal tract

An understanding of the physicochemical and physiological conditions in different regions of the GIT is needed to develop an effective *in vitro* method to test the potential gastrointestinal fate of nanoparticles (McClements and Li, 2010a, b; van Aken, 2010). Recently, there have been

proposals to standardize the *in vitro* methods used to test the gastrointestinal fate of foods and pharmaceutical preparations (Li et al., 2011; Minekus et al., 2014; Williams et al., 2012). These methods typically involve sequentially passing the sample through a series of artificial GIT fluids held at body temperature, *i.e.*, ≈ 37 °C (**Figure 12**). Initially, stock solutions of simulated saliva fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) are prepared with specified compositions and pH values (Minekus et al., 2014). In addition, stock solutions of the other major constituents in the gastrointestinal tract are prepared, such as digestive enzymes, bile salts, and calcium chloride. These additional solutions are mixed with the stock simulated fluids during the procedure to form the final SSF, SGF, and SIF fluids (**Table 7**). The sample to be tested is then passed sequentially through oral, gastric, and small intestine phases (**Figure 12**):

Oral: The sample (nanoparticle suspension) and final SSF (pH 7) are combined at a volume ratio of 1:1. For fluid samples this mixture is simply stirred for 2 minutes at a specified speed (*e.g.*, 60 s^{-1}), whereas for semi-solid or solid samples the mixture is blended for 2 minutes using a high shear mixer to simulate matrix disruption during mastication. The final SSF is a neutral aqueous solution ($\text{pH} \approx 7$) containing constituents that simulate human saliva, such as acids, buffers, minerals, biopolymers (mucin), and enzymes (amylase). The mixture resulting from the mouth phase is referred to as the bolus.

Stomach: The bolus sample is mixed with final SGF at a 1:1 volume ratio. For fluid samples the mixture is stirred for 2 hours at a specified speed (*e.g.*, 60 s^{-1}), whereas for semi-solid or solid samples, it may again be necessary to apply some kind of mechanical device to simulate the complex mechanical forces and fluid flows associated with gastric motility (Ferrua et al., 2011; Kong and Singh, 2011). Typically, the final SGF is a highly acidic aqueous solution

($\text{pH} \approx 3$) with a composition that simulates gastric conditions, *e.g.*, acids, buffers, salts, and digestive enzymes (proteases and lipases). The material collected at the end of the gastric processing stage is referred to as chyme.

Small Intestine: The chyme sample is combined with the final SIF at a 1:1 volume ratio and then stirred for 2 hours at a fixed speed (*e.g.*, 60 s^{-1}). Typically, the SIF is a neutral aqueous solution ($\text{pH} \approx 7$) containing various components that simulate small intestine composition, such as bile salts, phospholipids, bases, buffers, mineral salts, and digestive enzymes (proteases and lipases). The material collected at the end of the small intestinal processing stage is often referred to as the digest. The digest can be passed on to a simulated colon stage, used to analyze the release and absorption of bioactive components, and/or used to study changes in the structure and composition of the nanoparticles (**Figure 13**).

Colon: The digest is typically incubated in simulated colonic fluids (SCF) for a specified time and agitation conditions. The colon is one of the most difficult parts of the GIT to simulate in the laboratory because it requires the cultivation of colonic bacteria under anaerobic conditions (Yoo and Chen, 2006b). *In vitro* testing methods designed to simulate the colon range from simple static batch microbial cultures to multiple stage continuous cultures (Macfarlane and Macfarlane, 2007; Ouwehand et al., 2009; Rummey and Rowland, 1992). A sample is incubated in one or more SCFs containing bacteria representative of those typically found in the human colon. These bacteria may be cultivated from animal or human feces. One difficulty in accurately simulating the human colon is the considerable person-to-person variations in bacterial populations that exist. Rather than using bacteria, it is possible to formulate SCF containing a cocktail of enzymes typically produced by colonic bacteria *e.g.*, glycosidases to

degrade dietary fibers and proteases to degrade proteins (Souto-Maior et al., 2009; Yang, 2008).

Due to the difficulties in setting up and maintaining *in vitro* colonic models many researchers prefer to go directly to animal models (Macfarlane and Macfarlane, 2007). Alternatively, if the active ingredient and nanoparticles are fully digested and absorbed in the small intestine, then this step may be omitted.

Researchers can establish their own *in vitro* GIT models in the laboratory using appropriate reagents (such as acids, bases, buffers, minerals, bile salts, phospholipids, biopolymers, and enzymes) and equipment (such as glassware, stirrers, temperature control units, pH meters, and titration units). Nevertheless, it is highly advised for researchers to use one of the recently standardized methods so that results from different laboratories can be compared. In addition, sophisticated analytical instruments have been commercially developed to simulate the GIT process and these have been used in many studies, *e.g.*, the TIMTM system from TNO Quality of Life, The Netherlands (Minekus et al., 1999; Venema et al., 2009).

Absorption

Knowledge of the absorption of active ingredients and nanoparticles is often important for establishing the efficacy and toxicity of nanoparticle-based delivery systems. Typically, absorption occurs at the epithelium cells lining the inner walls of the small intestine, and can be simulated using physical, biological, or cell-culture models.

Physical Models: If the rate-limiting step in the uptake of a component by the human body is its solubility within the gastrointestinal fluids (rather than its absorption by the epithelium cells), then it can be assumed that the concentration of the component in the SIF in a soluble form is representative of the amount that will be absorbed by the epithelium cells. The amount

of a component solubilized within the SIF can be determined by measuring their aqueous phase concentration, either directly or after they have been isolated from the undigested material by centrifugation, filtration, or dialysis (Christensen et al., 2004; Do Thi et al., 2009). A simple method of isolating the digested component is to place the whole sample within a dialysis bag with a suitable pore size (to prevent undigested material from exiting), and then measuring the amount of released material that moves across the semi-permeable membrane using an appropriate analytical method.

Ex vivo Permeation Methods: In these methods, a section of gastrointestinal tract is excised from an animal after it has been sacrificed, and is then washed to remove extraneous components. The intestinal section is then clamped between two chambers: one that contains the sample to be analyzed (donor chamber) and another that contains buffer solution (receiver chamber) (Dahan and Hoffman, 2007). The transport of nanoparticles or active ingredients across the chamber is then measured over time using suitable analytical methods. Alternatively, the sample is placed inside an intact section of GIT that is clamped at the ends and then placed in a buffer solution. The amount of active component or nanoparticles that move across the intestinal walls and into the external buffer solution is then measured using an appropriate analytical method.

Cell Culture Methods: Cell culture models are widely used to simulate the epithelium cells coating the inner surface of the small intestine, where the absorption of active components and nanoparticles normally occurs. Caco-2 cells are the most commonly used cell culture models for studying the absorption of active components and nanoparticles (Dhuique-Mayer et al., 2007; Reboul et al., 2006; Versantvoort et al., 2003). A layer of Caco-2 cells is normally grown directly

on the surface of a plastic plate (such as a petri dish) or on a semi-permeable membrane placed within a petri dish (**Figure 13**). An aqueous suspension of the sample (which may previously have been exposed to simulated GIT conditions) is placed in contact with the model epithelium cells, and the amount of active component either absorbed by the cells or that passes through the cells is measured. A suitable analytical technique is used to measure the location or quantity of absorbed material, *e.g.*, microscopy, chromatography, spectrometry, electrophoresis, or wet chemistry methods. An example of a microscopy approach to study the absorption of gold nanoparticles by Caco-2 cells is shown in **Figure 14**.

We recommend that a standardized Caco-2 cell culture model be used to analyze the absorption of active ingredients or nanoparticles (Vors et al., 2012). We also stress that it is important to pass the sample through the full GIT model prior to applying it to the Caco-2 cells because passage through the mouth, stomach, and small intestine may cause appreciable changes in particle properties that influence the absorption of components. Either the entire digest resulting from the small intestine phase or only the micelle phase collected can be incubated with the Caco-2 cells. Often it is necessary to dilute the samples extensively prior to incubation, otherwise some of the components in the digest may inactive the Caco-2 cells, *e.g.*, high bile salt or surfactant concentrations.

***In Vivo* Approaches**

In vitro methods are useful for rapidly screening formulations with different compositions and structures, and for establishing the physicochemical basis of the gastrointestinal fate of nanoparticles (McClements and Li, 2010a). However, they are insufficiently sophisticated to mimic the complex processes that occur within the human GIT. A more realistic indication of

the biological fate of nanoparticles can be obtained using *in vivo* studies with animals and/or humans (McClements et al., 2009a). Numerous types of information can be obtained from animal feeding studies. An animal may be fed the nanoparticles being analyzed, either alone or as part of a complex diet, over a specified period. Changes in the whole animal (such as behavior or body weight) or changes in specific internal organs (such as gastrointestinal tract, liver, pancreas, kidneys, lungs, heart, spleen, brain, and adipose tissue) may be measured, typically after the animal is sacrificed. Alternatively, changes in the composition of the blood, breath, feces, and/or urine of an animal may be measured to monitor the absorption, metabolism, and/or excretion of a particular active component or its metabolites. In some cases, it is possible to use invasive tubes to collect samples as nanoparticle suspensions pass through the digestive tract of live animals. Alternatively, whole-body imaging methods, such as those based on magnetic resonance, ultrasound, X-ray, or fluorescence, can be used to visualize the processes occurring within the GIT as a material passes through. The *in vivo* approach typically provides a more accurate representation of how nanoparticles perform in practice, but they have many limitations since they are expensive, time-consuming, and have ethical and legal implications. It is also important to note that nanoparticles may behave differently in an animal's GIT than in a human's GIT.

***In vitro* versus *In vivo* Correlations**

In vitro studies offer several advantages over *in vivo* studies for testing nanoparticle suspensions, because they are usually faster, cheaper, more versatile, and provide more details about physicochemical mechanisms (Dahan and Hoffman, 2006; Porter and Charman, 2001; Yoo and Chen, 2006a). Nevertheless, it is extremely difficult to accurately mimic the complex

physicochemical and physiological processes that occur within the human GIT. For this reason, it is useful to combine *in vitro* and *in vivo* studies when developing an appropriate nanoparticle-based delivery system. The *in vitro* method is used to rapidly screen a range of initial samples, and then the *in vivo* method is used to test those candidates that are the most promising. If this approach is used it is important to establish *in vitro – in vivo* correlations (IVIVC) so that an *in vitro* method can be used give reliable predictions of the performance of nanoparticles *in vivo* (Dahan and Hoffman, 2008; Porter and Charman, 2001; Pouton and Porter, 2006). Typically, the rate and/or extent of absorption of an active ingredient or nanoparticle is measured for similar test samples using an *in vitro* method and an *in vivo* method, and then the results are correlated to one another (Li et al., 2012a). Computational models are particularly useful for predicting the real-life performance of nanoparticles based on knowledge of their composition and structure, as well as physiological conditions (Dressman et al., 2011; Mathias and Crison, 2012).

Measurement of Changes in Nanoparticle Properties in GIT

The composition, structure, and/or physicochemical properties of nanoparticles can be measured at specific points in actual or simulated GIT environments to establish the major factors influencing their gastrointestinal fate. The various analytical techniques mentioned earlier can be used for this purpose *e.g.*, microscopy, particle size analysis, charge measurements, stability.

Case Study: Potential gastrointestinal fate of lipid nanoparticles

A specific example of the study of the gastrointestinal fate of food-grade nanoparticles is provided to demonstrate the utility of a standardized approach of characterizing their properties.

At a minimum, we recommend that nanoparticles should be tested using a standardized *in vitro* gastrointestinal tract (GIT) model that simulates the mouth, stomach, and small intestine (**Figure 12**). The mean size, size distribution, electrical charge, and aggregation state of the particles should be measured initially and after each stage of the GIT model (**Table 8, Figure 15A**). In addition, the hydrolysis of any digestible components used to assemble the nanoparticles should be measured at appropriate GIT stages, *e.g.*, starch hydrolysis within the mouth, and protein and lipid hydrolysis in the stomach and small intestine (**Figure 15B**). Finally, the release and stability of any encapsulated active ingredients should be measured throughout the simulated GIT using suitable analytical methods, such as wet chemistry, chromatography, spectrophotometry, and electrophoresis methods (**Figure 15C**). The utilization of these standardized procedures would allow different types of nanoparticles to be compared under similar conditions.

CONCLUSIONS

There has been a rapid increase in the development and application of food-grade nanoparticles over the past decade or so. At present, it is difficult to compare the relative advantages and disadvantages of different kinds of nanoparticles because researchers use different analytical techniques and experimental protocols to characterize them. In this article, different methods of characterizing the most important properties of food-grade nanoparticles have been critically evaluated, such as composition, morphology, size, charge, and stability. This information was then used to propose a number of standardized protocols for measuring particle properties (**Figure 16**), and for evaluating their response to environmental stresses (**Table 7**) and

gastrointestinal conditions (**Figure 12, Table 8**) that they may experience during their application. The widespread use of these methods would facilitate the rational comparison of the benefits and limitations of different kinds of nanoparticles for specific applications.

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Table 1: Selected examples of different kinds of food-grade nanoparticles reported in the recent literature. There are large variations in particle composition, size, and charge depending on ingredients and preparation methods used. These references were found through a literature search using the keywords “food” and “nanoparticles” using Web of Science (June 2014). Key: ND = Not determined; FFFF = Flow field-flow fractionation; ME = micro-electrophoresis; DLS = dynamic light scattering; SLS = static light scattering.; NTA = nanoparticle tracking analysis. pH is given in square brackets and measurement method is given in round ones.

Nanoparticle Core	Nanoparticle Shell	Structure				Reference
		Particle Diameter	Particle Charge	Composition	Morphology	
Carbohydrate	-	$d_Z = 180 \text{ nm}$ [pH 4.2] (DLS)	+39.3 mV [pH 4.2] (ME)	Chitosan & sodium tripolyphosphate	Spherical (TEM)	(Solval et al., 2014)
Lipid	Surfactant	$d_{\text{Z}} = 116 \text{ to } 178 \text{ nm}$ $d_{\text{Z}} = 143 \text{ to } 189 \text{ nm}$ [pH 7.0 – 9.5] (SLS)	ND	SLNs stabilized by bile salts, amino acids and/or ionic surfactants.	ND	(Salminen et al., 2014)
Lipid	Surfactant	$d_Z = 294 \text{ nm}$ [pH 2] $d_Z = 189 \text{ nm}$ [pH 12] (DLS)	-1.2 mV [pH 2] -20.0 mV [pH 12] (ME)	SLNs stabilized by Tween 80.	ND	(Choi et al., 2014)
Lipid	Surfactant	$d_Z = 370 \text{ to } 660 \text{ nm}$ (DLS)	-38.0 to -39.1 mV (ME)	Rosmarinic acid loaded SLNs stabilized by Tween 80.	ND	(Campos et al., 2014)
Lipid	Surfactant	$d_{\text{Z}} = 113 \text{ to } 153 \text{ nm}$ $d_{\text{Z}} = 129 \text{ to } 587 \text{ nm}$ [pH 7] (SLS)	ND	Fish oil loaded SLNs stabilized by lecithin and bile salts.	ND	(Salminen et al., 2013)
Protein	Protein	$d_Z = 350 \text{ nm}$ [pH 7] (DLS)	$\approx +15 \text{ mV}$ [pH 3] $\approx -15 \text{ mV}$ [pH 7]	Tangeretin-loaded protein nanoparticles	Spherical (TEM)	(Chen et al., 2014)

			(ME)	fabricated from zein and β -lactoglobulin.		
Lipid	Protein	$d_z = 77.8 \text{ nm}$ [pH 7.0] (DLS)	-35.7 mV [pH 7] (ME)	β -carotene loaded protein nanoparticles stabilized with sodium caseinate.	Non-spherical (TEM)	(Yi et al., 2014)
Lipid	Protein	$d_z = 89.7 \text{ nm}$ [pH 7.0] (DLS)	-29.9 mV [pH 7] (ME)	β -carotene loaded lipid nanoparticles stabilized with whey protein isolate.	Spherical (TEM)	(Yi et al., 2014)
Lipid	Protein	$d_z = 372 \text{ nm}$ [pH 7.0] (DLS)	-38mV [pH 7.0] (ME)	β -carotene loaded lipid nanoparticles stabilized with soy protein isolate.	Spherical (TEM)	(Yi et al., 2014)
Protein-Carbohydrate	-	$d_z = 109 \text{ to } 200 \text{ nm}$ [pH 6] (DLS)	ND	Caffeine loaded biopolymer (pectin and WPI) nanoparticles	Spheroid (SEM)	(Gazme and Madadlou, 2014)
Protein	-	$d_z = 25 \text{ to } 164 \text{ nm}$ [pH 7.2] (DLS)	ND	α -lactalbumin nanoparticles	ND	(Dhayal et al., 2014)
Protein	Protein	$d_{\overline{z}} = 122 \text{ to } 136 \text{ nm}$	-33 to -36 mV	Zein nanoparticles	Spherical	(Chen and

		[pH 8 - 11] (DLS)	[pH 8 - 11] (ME)	coated with sodium caseinate	(TEM)	Zhong, 2014)
Carbohydrate	-	$d_{\text{Z}} = 360 \text{ nm}$ [pH 6.5] (DLS)	-21.6 mV [pH 6.5] (ME)	Starch nanoparticles.	ND	(Tan et al., 2014)
Carbohydrate	-	$d_{\text{Z}} = 31 \pm 5 \text{ nm}$ (DLS)	ND	Starch nanoparticles (Cassava)	Spheroid (TEM)	(Lamanna et al., 2013)
Carbohydrate	-	$d_{\text{Z}} = 41 \pm 7 \text{ nm}$ (DLS)	ND	Starch nanoparticles (Waxy maize)	Not Spherical (TEM)	(Lamanna et al., 2013)
Carbohydrate	-	$d_{\text{Z}} = 555 - 77 \text{ nm}$ [pH 6 & 7.4] (DLS)	ND	Cationic starch nanoparticles. With varying amounts of starch.	Spheroid (TEM)	(Huang et al., 2013)
Carbohydrate	-	$d_{\text{Z}} = 79 - 250 \text{ nm}$ (DLS)	+48 to +470 mV (ME)	Cationic starch nanoparticles.	Spherical (TEM)	(Huang et al., 2013)
Carbohydrate	-	$d = 88.8 \text{ nm}$ [pH 4.8] (TEM)	$\approx + 23 \text{ mV}$ [pH 4]	Chitosan nanoparticles.	Spherical (TEM)	(Martelli et al., 2013)
Mineral	-	$d = 50 - 400 \text{ nm}$	ND	Zero-valent selenium	Spherical or	(Lampis et

		(SEM)		nanoparticles.	oblong (SEM)	al., 2014)
Mineral	Carbohydrat e	d = 64 nm [pH 2 – 7] (FIFFF)	-14.2 to -20.4 mV [pH 2 – 7] (ME)	Selenium nanoparticle stabilized with pectin.	Spherical (TEM)	(Pornwilar d et al., 2014)
Mineral	Protein	d = 30 nm [pH 2 – 7] (FIFFF)	-37.9 to -20.4 mV [pH 2 - 7] (ME)	Selenium nanoparticles stabilized with ovalbumin.	Spherical (TEM)	(Pornwilar d et al., 2014)
Mineral	-	d = 70 – 120 nm [pH 7.2] (TEM)	ND	Selenium nanoparticles	Spherical (TEM)	(Zhang et al., 2012)
Carbohydrate	Carbohydrat e	d = 200 to 2000 nm [pH 6] (SEM)	ND	Calcium alginate nanoparticles.	Spherical (SEM)	(Paques et al., 2014)
Carbohydrate	-	d = 6 to 400 nm [pH 4 – 10] (SEM)	ND	Calcium alginate nanoparticles.	Spherical (Cryo-SEM)	(Paques et al., 2013)
Inorganic	-	d ≈ 50 nm [pH 7.3] (TEM & SEM)	ND	Zinc oxide nanoparticles	Spherical (TEM & (SEM)	(Akbar and Anal, 2014)
Inorganic	-	d = 103 nm	ND	Titanium dioxide	Non-	(Gitrowski

		(TEM & NTA)		nanoparticles	spherical (TEM)	et al., 2014)
Inorganic	-	$d_z = 36 \text{ nm}$ (DLS & FFFF)	-9.7 mV	Silver nanoparticles	ND	(Oliver et al., 2014)
Inorganic	-	$d = 36 \text{ or } 117 \text{ nm}$ (DLS)	ND	Silver nanoparticles	Non-spherical (SEM & TEM)	(Kim and Shin, 2014)
Inorganic	-	$d = 66 \text{ nm [pH 7]}$ (TEM & NTA)	-30.7 [pH 7]	Silver nanoparticles	Spherical (TEM)	(Gade et al., 2014)
Lipid	Surfactant	$d_z = 60\text{-}170 \text{ nm [pH 7]}$ (DLS)	-5 to -20 mV [pH 7] (ME)	Tween 20 coated corn oil lipid nanoparticles.	Spherical (TEM)	(Troncoso et al., 2012)
Lipid	Surfactant	$d_z = 25 \text{ nm [pH 3.5]}$ (DLS)	ND	Tween coated lipid (orange oil and MCT) nanoparticles.	ND	(Chang and McClements, 2014)
Lipid	Surfactant	$d_z = 88 \text{ nm [pH 2-8]}$ (DLS)	$\approx +2 \text{ to } -36 \text{ mV}$ [pH 2-8]	Sucrose monopalmitate coated lipid (lemon oil) nanoparticles.	ND	(Rao and McClements, 2011)
Lipid	Surfactant	$d_z = 150 \text{ to } 240 \text{ nm}$	ND	Tween coated lipid	ND	(Chang et

		[pH 3.5] (DLS)		(thyme oil and MCT) nanoparticles.		al., 2012)
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Table 2: Summary of analytical methods suitable for establishing the composition of nanoparticles.

Component	Methods
Protein	
- Total	Measure total nitrogen (<i>e.g.</i> , Kjeldahl, Dumas); UV-visible methods (<i>e.g.</i> , direct, Biuret, Lowry ...)
- Type	SDS-PAGE; Size exclusion chromatography; Biochemical (Enzyme-based assays); Mass spectrometry
Lipid	
- Total	Solvent extraction methods (<i>e.g.</i> , Soxhlet); Non-solvent extraction methods (<i>e.g.</i> , Gerber, Babcock, detergent methods)
- Type	Chromatography (TLC, GC, HPLC); Spectroscopy (UV-visible, NMR, IR, Raman); Mass spectrometry
Carbohydrate	
- Total	Wet-chemistry methods (<i>e.g.</i> , colorimetric, gravimetric or titration)
- Type	Chromatography (TLC, GC, HPLC), Biochemical (enzyme or antibody assays); Mass spectrometry
Mineral	
- Total	Measure ash content (<i>e.g.</i> , muffle furnace, wet ashing)
- Type	Wet-chemistry methods (<i>e.g.</i> , colorimetric, gravimetric or titration); Spectroscopy methods (<i>e.g.</i> , atomic emission or absorption, X-ray)

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Table 3. Different methods of expressing the concentration of nanoparticles. It is always important to clearly indicate which concentration parameter is being used when reporting data.

Concentration Parameter	Definition	Typical units
Number concentration	$C_N = N_{NP}/V_T$	m^{-3}
Volume concentration	$C_V = V_{NP}/V_T$	Dimensionless
Mass concentration	$C_M = M_{NP}/V_T$	$kg\ m^{-3}$ or $mg\ mL^{-1}$
Mass fraction	$\phi_M = M_{NP}/M_T$	Dimensionless
Weight percentage	$\% \phi_M = 100 \times M_{NP}/M_T$	wt.% or w/w %
Volume fraction	$\phi_V = V_{NP}/V_T$	Dimensionless
Volume percentage	$\% \phi_V = 100 \times V_{NP}/V_T$	vol. % or v/v %

Symbols: n is number, V is volume, m is mass; **Subscripts:** NP is nanoparticle, T is total.

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Table 4. Different ways of expressing the mean particle diameter of nanoparticles. It is always important to specify which mean diameter is given when reporting data. Adopted from McClements (2014).

Type of Mean	Symbol	Definition	Quantity Averaged	Weighting-Factor
Number-length mean diameter	d_{NL} or d_{10}	$d_{10} = (\sum n_i d_i) / (\sum n_i)$	Diameter	Number in Class
Number-area mean diameter	d_{NA} or d_{20}	$d_{20} = [(\sum n_i d_i^2) / (\sum n_i)]^{1/2}$	Surface Area	Number in Class
Number-volume mean diameter	d_{NV} or d_{30}	$d_{30} = [(\sum n_i d_i^3) / (\sum n_i)]^{1/3}$	Volume	Number in Class
Area-volume mean diameter	d_{AV} or d_{32}	$d_{32} = (\sum n_i d_i^3) / (\sum n_i d_i^2)$	Diameter	Area in Class
Volume-length mean diameter	d_{VL} or d_{43}	$d_{43} = (\sum n_i d_i^4) / (\sum n_i d_i^3)$	Diameter	Volume in Class
Z-average	d_Z or $\approx d_{65}$	$d_{65} \approx (\sum n_i d_i^6) / (\sum n_i d_i^5)$	Diameter	Intensity

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Table 5: Suggested environmental stress tests for establishing the stability range of food-grade nanoparticles. The most important stress tests that should be carried out are highlighted in bold, whereas the more product-specific ones are highlighted in italics.

	Particle Diameter	Particle Charge	Particle Stability	Comments
Initial values				Report initial size, charge, & stability of prepared nanoparticles.
Long term stability				Report size, charge & stability before and after storage (6 months, 5, 25 and 37 °C)
pH				Report size, charge, and stability at pH 2-8
Ionic Strength				Report size, charge, and stability at 0 – 500 mM NaCl and 0 – 100 mM CaCl ₂
Thermal processing				Report size, charge, & stability after exposure to temperatures from 30 to 90 °C for 30 min
<i>Freeze-thaw</i>				Report size, charge, and stability after freezing at -20 °C for 22 hour & thawing at 20°C for 2 hour
<i>Dehydration</i>				Report size, charge, and stability after dehydration and rehydration
<i>Light exposure</i>				Report size, charge, and stability after exposure to light for 1 week.
<i>Mechanical Stability</i>				Report size, charge, & stability after exposure to high shear

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				mixer for 2 minutes.
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Table 6: Characterization of the key physicochemical and structural properties of β -lactoglobulin-coated lipid nanoparticles in oil-in-water nanoemulsions. ND = not determined in this study. The lipid phase contains a 1:1 mixture of vitamin E acetate and lemon oil for most reported data, with some exceptions noted in the text.

	Stress Conditions	Particle Diameter	Particle Charge	Morphology
Initial values (at pH 7, 25 °C)	None	$d_{32} = 110$ nm	-48 nm	Non-aggregated spheroids (see Figure)
Long term stability (at pH 7, for 6 months)	5 °C	ND	ND	ND
	25 °C	ND	ND	ND
	37 °C	ND	ND	ND
pH (at 25 °C)	pH 2	$d_{32} = 110$ nm	+39 mV	Non-aggregated
	pH 3	$d_{32} = 110$ nm	+48 mV	Non-aggregated
	pH 4	$d_{32} = 111$ nm	+32 mV	Non-aggregated
	pH 5	$d_{32} = 13,000$ nm	0 mV	Flocculated
	pH 6	$d_{32} = 110$ nm	-31 mV	Non-aggregated
	pH 7	$d_{32} = 110$ nm	-48 mV	Non-aggregated
	pH 8	$d_{32} = 109$ nm	-45 mV	Non-aggregated
Ionic Strength (at pH 7, 25 °C)	0 mM NaCl	$d_{32} = 111$ nm	-54 mV	Non-aggregated
	100 mM NaCl	$d_{32} = 111$ nm	-48 mV	Non-aggregated

	200 mM NaCl	$d_{32} = 113 \text{ nm}$	-48 mV	Non-aggregated
	300 mM NaCl	$d_{32} = 115 \text{ nm}$	-47 mV	Some flocculation
	400 mM NaCl	$d_{32} = 118 \text{ nm}$	-46 mV	Some flocculation
	500 mM NaCl	$d_{32} = 118 \text{ nm}$	-45 mV	Some flocculation
Thermal processing (at pH 7, 150 mM NaCl for 30 min)	30 °C	$d_{32} = 178 \text{ nm}$	-45 mV	Non-aggregated
	40 °C	$d_{32} = 167 \text{ nm}$	-47 mV	Non-aggregated
	50 °C	$d_{32} = 211 \text{ nm}$	-44 mV	Minor flocculation
	60 °C	$d_{32} = 393 \text{ nm}$	-40 mV	Minor flocculation
	70 °C	$d_{32} = 51,000 \text{ nm}$	ND	Large flocculation
	80 °C	$d_{32} = 52,000 \text{ nm}$	ND	Large flocculation
	90 °C	$d_{32} = 52,000 \text{ nm}$	ND	Large flocculation

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Table 7: Standardized *in vitro* digestion model that simulates the oral, stomach and small intestinal phases of the GIT based on Minekus et al (Minekus et al., 2014). Details about the compositions of the stock SSF, SGF, SIF, pancreatin, bile, and enzyme solutions are described in this reference.

Oral Phase	Gastric Phase	Intestinal Phase
5.0 mL Sample	10 mL Bolus sample	20 mL Chyme sample
3.5 mL Stock SSF	7.5 mL Stock SGF	11 mL Stock SIF
0.5 mL α -amylase (1500 U mL^{-1}) solution	1.6 mL pepsin (25000 U mL^{-1}) solution	5 mL pancreatin (800 U mL^{-1}) solution
0.025 mL CaCl_2 solution (300 mM)	0.005 mL CaCl_2 solution (300 mM)	2.5 mL fresh bile (160 mM) solution
0.975 mL distilled water	0.2 mL HCl solution (1000 mM)	0.04 mL CaCl_2 solution (300 mM)
	0.695 mL distilled water	0.15 mL NaOH solution (1000 mM)
		1.31 mL distilled water
pH 7.0	pH 3.0	pH 7.0

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Table 8: Example of a table that can be used to report the gastrointestinal fate of food-grade nanoparticles. Examples are given for sucrose monopalmitate (SMP)-coated lipid nanoparticles with a corn oil core. Data from Rao et al (Rao et al., 2013). Particle size distributions and microscopy images after each stage of digestion should also be reported, as well as digestion profiles (pH stat) and bioaccessibility data if an encapsulated component is present.

SMP-coated corn oil nanoparticles			
	Particle Diameter (d_{32})	Particle Charge (ζ)	Comments
Initial values (pH 7)	156 nm	-47.0	Nanoparticles are initially negatively charged and stable to aggregation
Mouth (pH 7)	170 nm	-32.6	Extensive droplet flocculation occurred within simulated oral conditions
Stomach (pH 3)	209 nm	-25.5	Some droplet flocculation occurred within simulated gastric conditions
Small Intestine (pH 7)	5300 nm	-25.4	Extensive droplet aggregation and digestion occurred under simulated intestinal conditions
Colon (pH 6)	ND	ND	Nanoparticles were completely digested after intestinal phase

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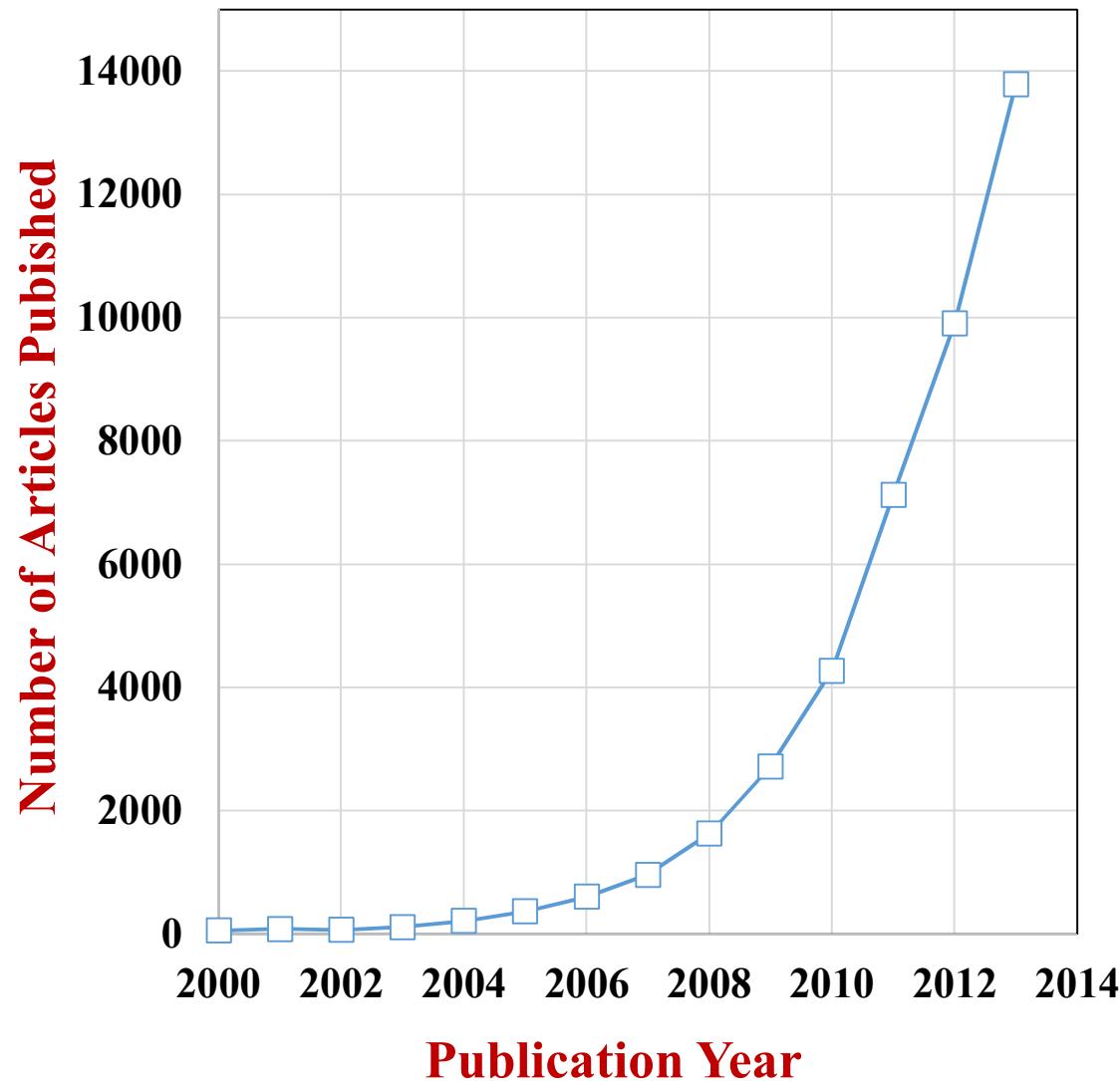


Figure 1: Number of scientific articles published with the key words “nanoparticles” and “food” in the past decade or so.

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Researched on Web of Science (Thomson Scientific) on June 2014.

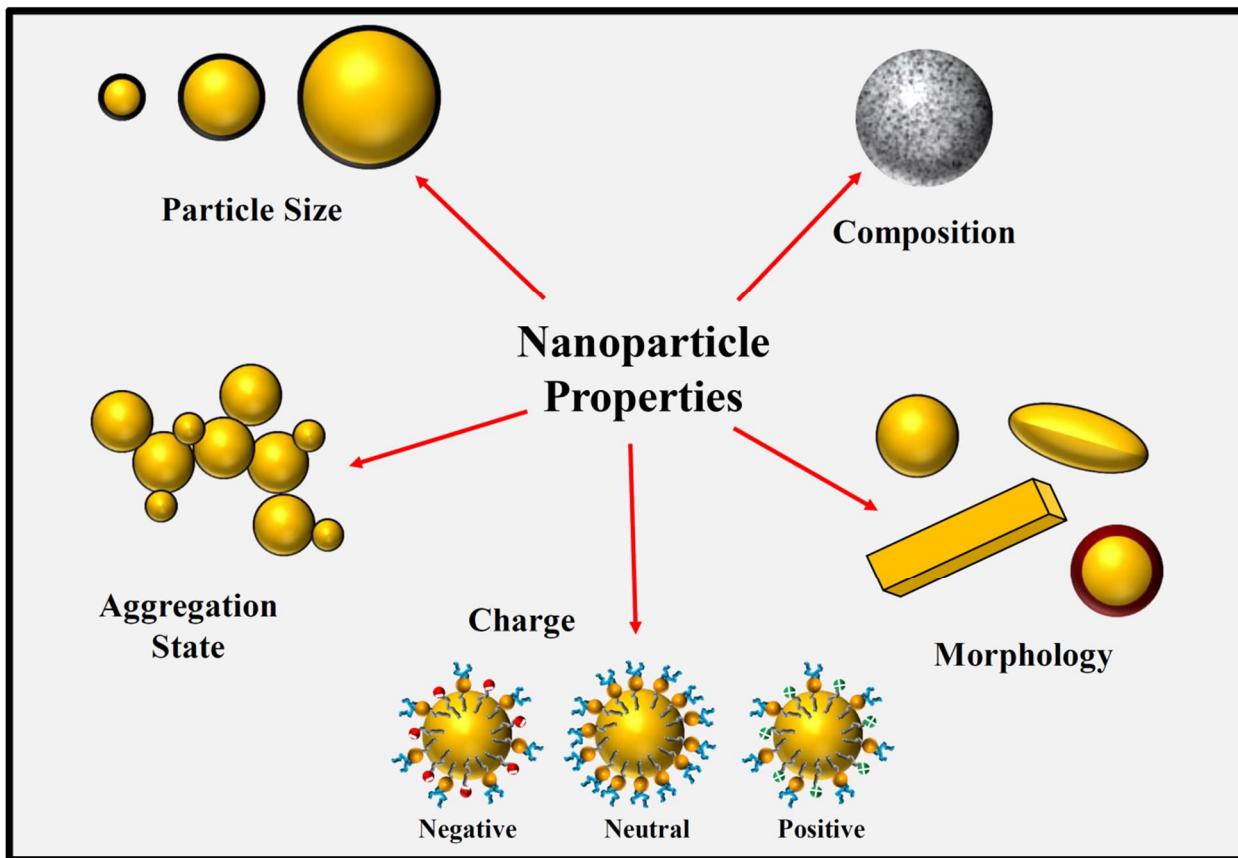


Figure 2: Food-grade nanoparticles vary according to their composition, size, charge, physical state, and morphology..

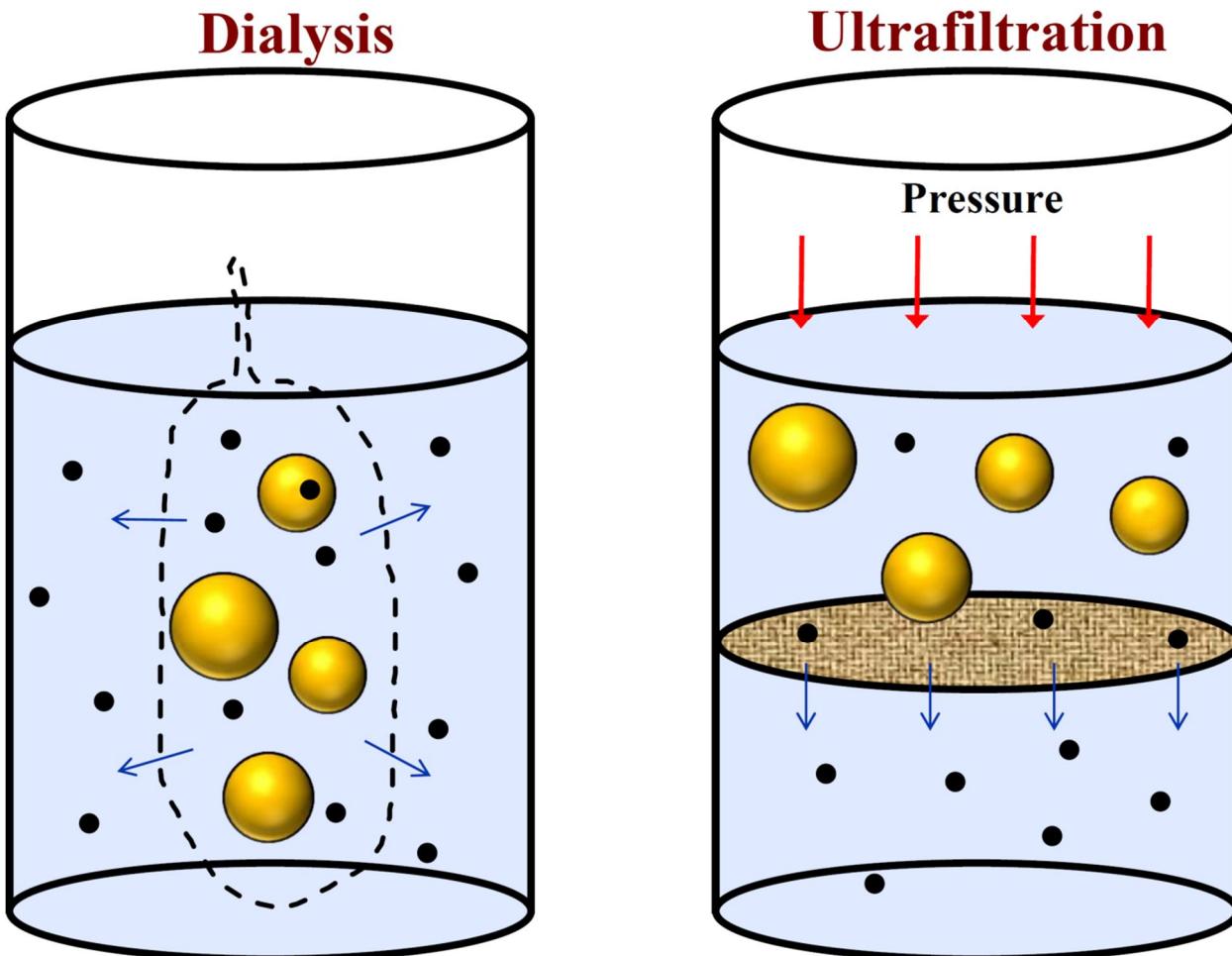


Figure 3. Nanoparticles can be separated from other particulate or polymeric matter using semi-permeable membranes, such as dialysis, filtration or ultrafiltration. Nanoparticles can be separated from small solutes by using a pore size smaller than the nanoparticle (as shown). Alternatively, they may be separated from large particles using a pore size larger than the

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nanoparticles.

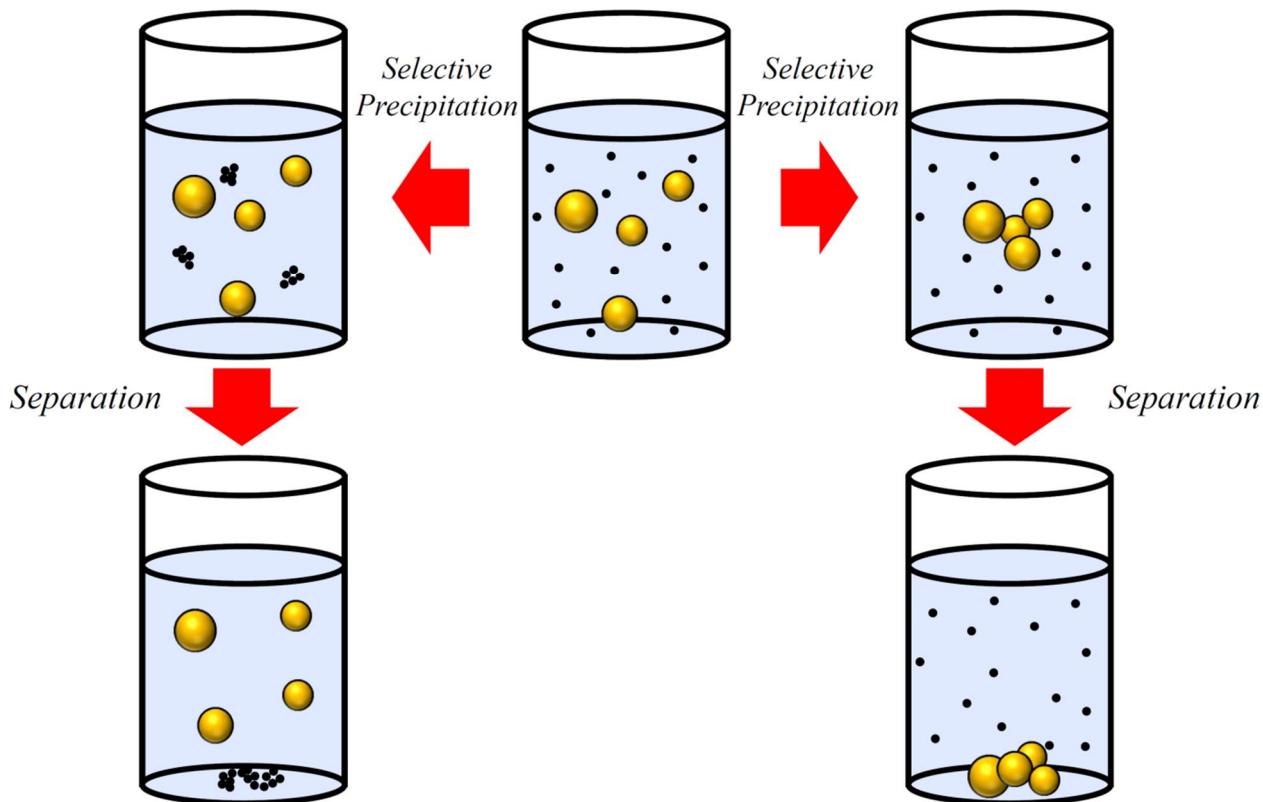


Figure 4. Nanoparticles can be separated from other particulate or polymeric matter using selective precipitation.

Environmental conditions (such as pH, ionic strength, solvent quality or temperature) are altered to selecting precipitate the nanoparticles or components in their surroundings.

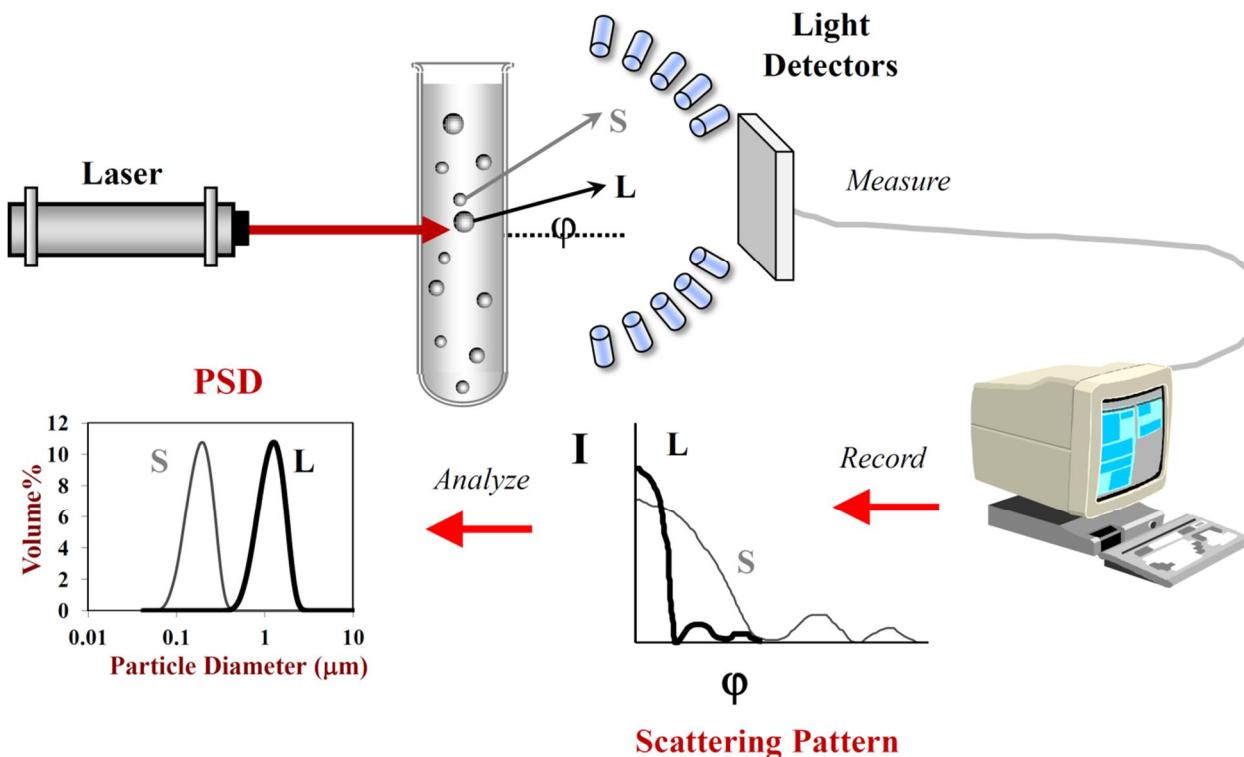


Figure 5: Particle size distribution of colloidal dispersions can be measured using static light scattering, which relies on determining the scattering pattern (intensity *versus* scattering angle), and using a mathematical model to convert the data into a particle size distribution

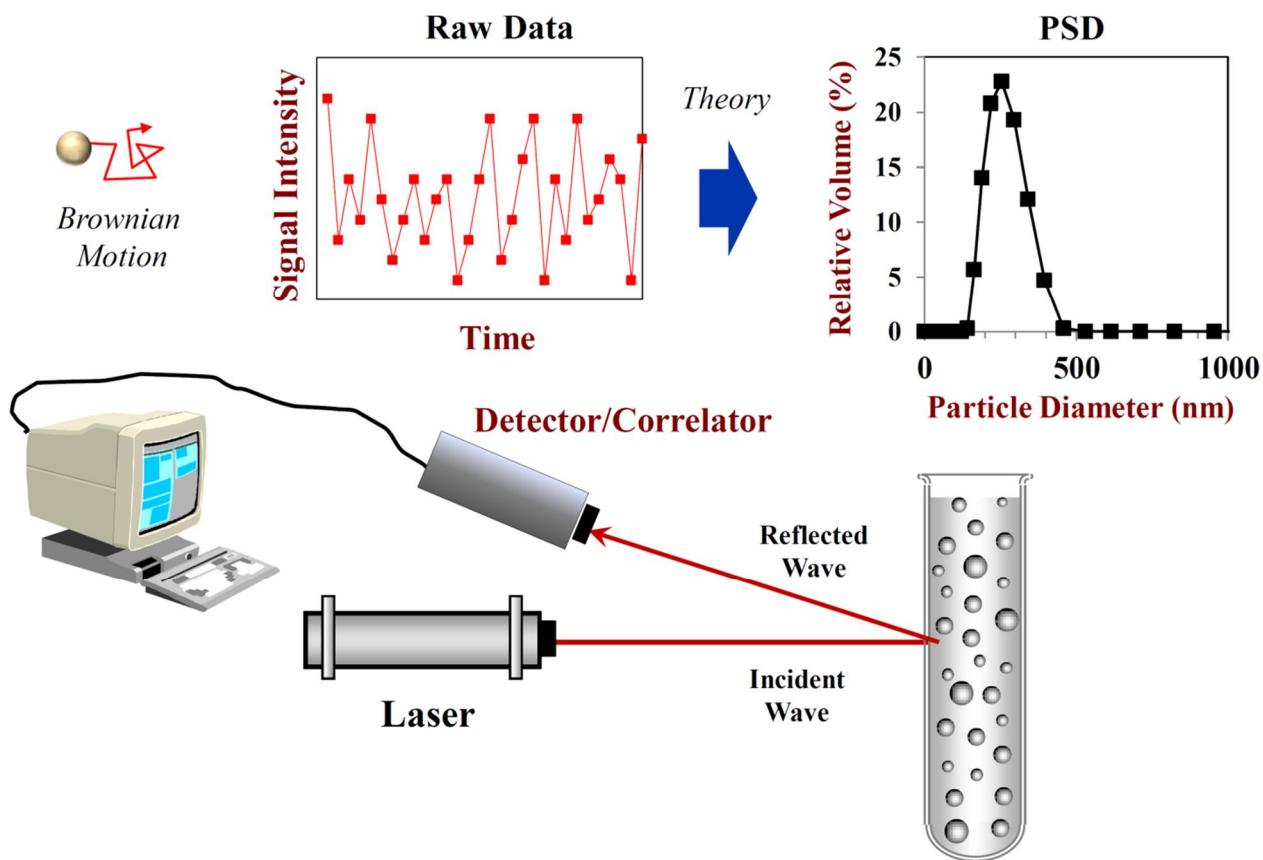


Figure 6: Particle size distributions of nanoparticle suspensions can be measured using dynamic light scattering, which relies on measuring fluctuations in scattered light, and using a mathematical model to convert the data into a particle size distribution. The PSD shown is of β -lactoglobulin-coated zein nanoparticles suspended in an aqueous buffer solution (pH 7.0, 10 mM PBS, ≈ 25 °C) measured using DLS.

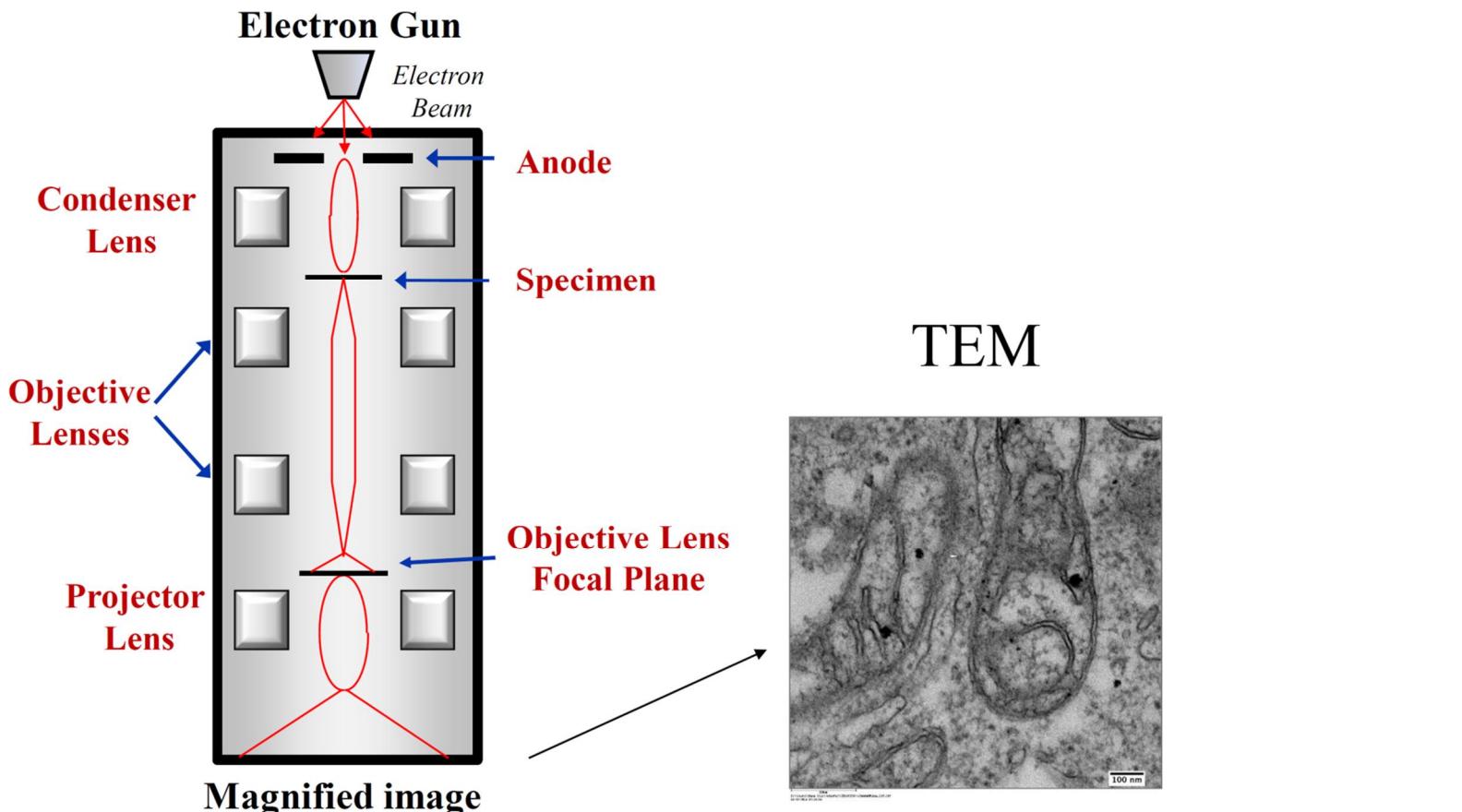


Figure 7: Schematic diagram of a transmission electron microscope (TEM) that can be used to provide images of the morphology and organization of nanoparticles. An image is formed by passing a beam of electrons through the specimen. The TEM image shown is of gold nanoparticles adsorbed by Caco 2 cells (Kindly supplied by Olivia Yao).

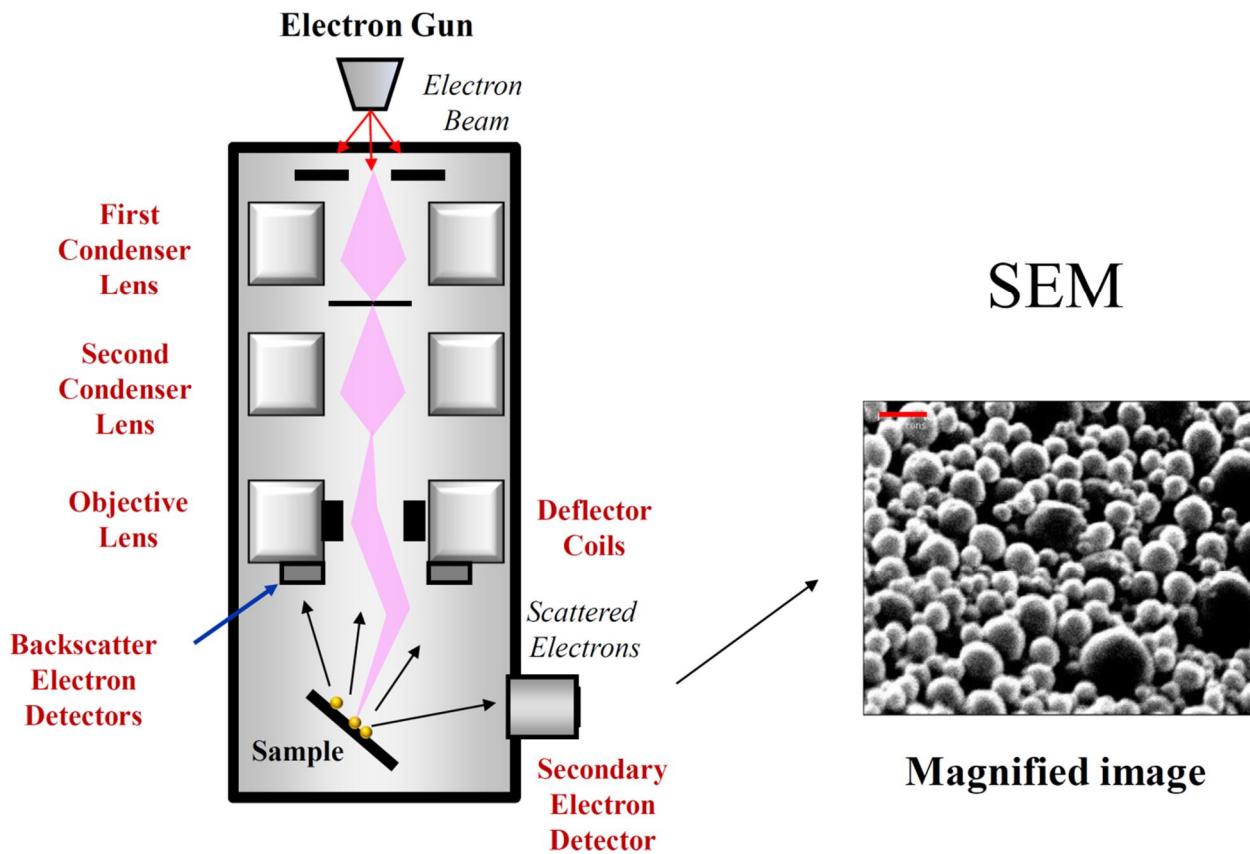


Figure 8: Schematic diagram of a scanning electron microscope (SEM) that can be used to provide images of the morphology and organization of nanoparticles. An image is formed by detecting the electrons scattered by a sample. The SEM image shown is of hydrocarbon oil droplets in an oil-in-water emulsion, with a scale bar of 1 mm (Image kindly supplied by John Coupland).

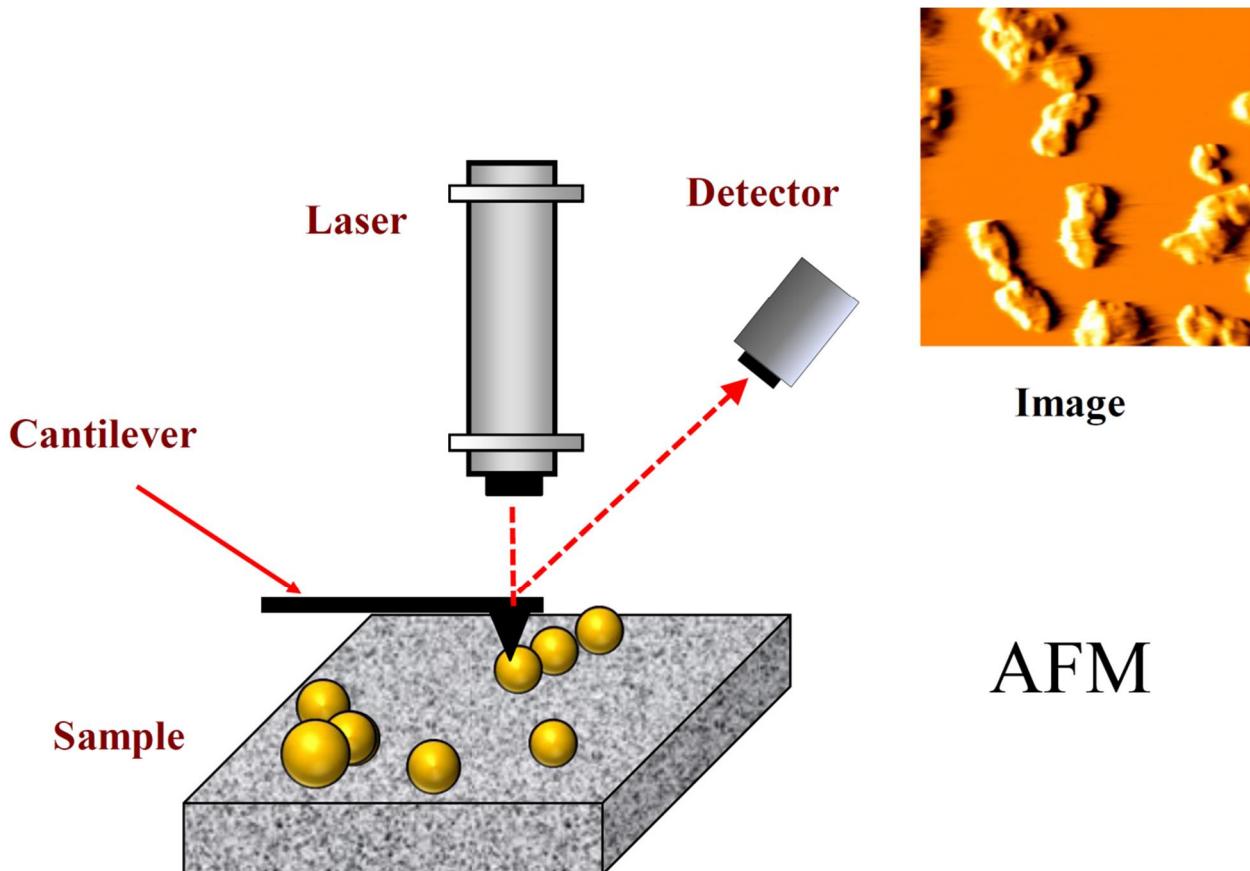


Figure 9: Schematic diagram of an atomic force microscope (AFM) that can be used to provide images of the structure of nanoparticle suspensions. The AFM image shown is of biopolymer nanoparticles produced by heating protein and polysaccharide complexes together (kindly supplied by Owen Jones). The image is 1000 nm by 1000 nm in dimensions.

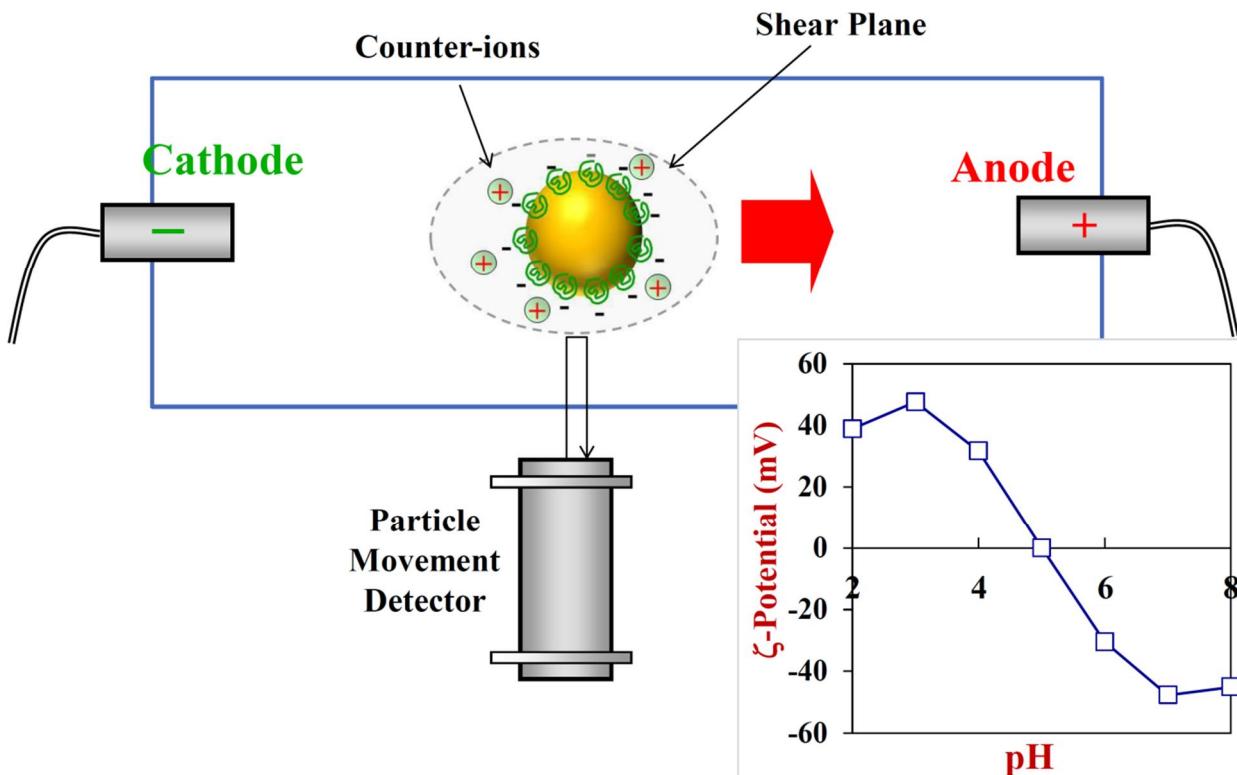


Figure 10: The electrical charge of food-grade nanoparticles can be determined using micro-electrophoresis. The direction and velocity of particle movement is measured used a suitable technique in a well-defined electrical field. Particle charge characteristics can be represented as z-potential versus pH.

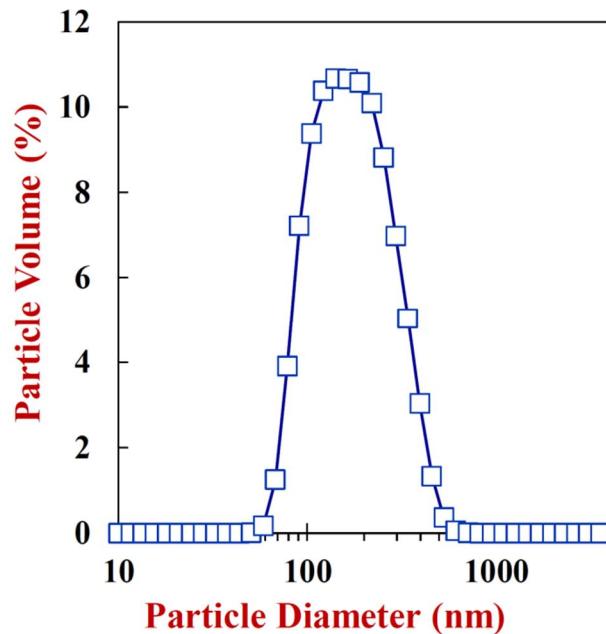
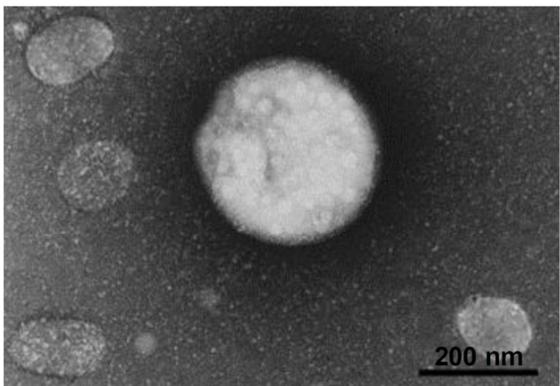


Figure 11. Transmission electron microscopy (TEM) image and particle size distribution (measured by DLS) of b-lactoglobulin coated lipid nanoparticles in a corn oil-in-water nanoemulsion. (Image from Elizabeth Troncoso).

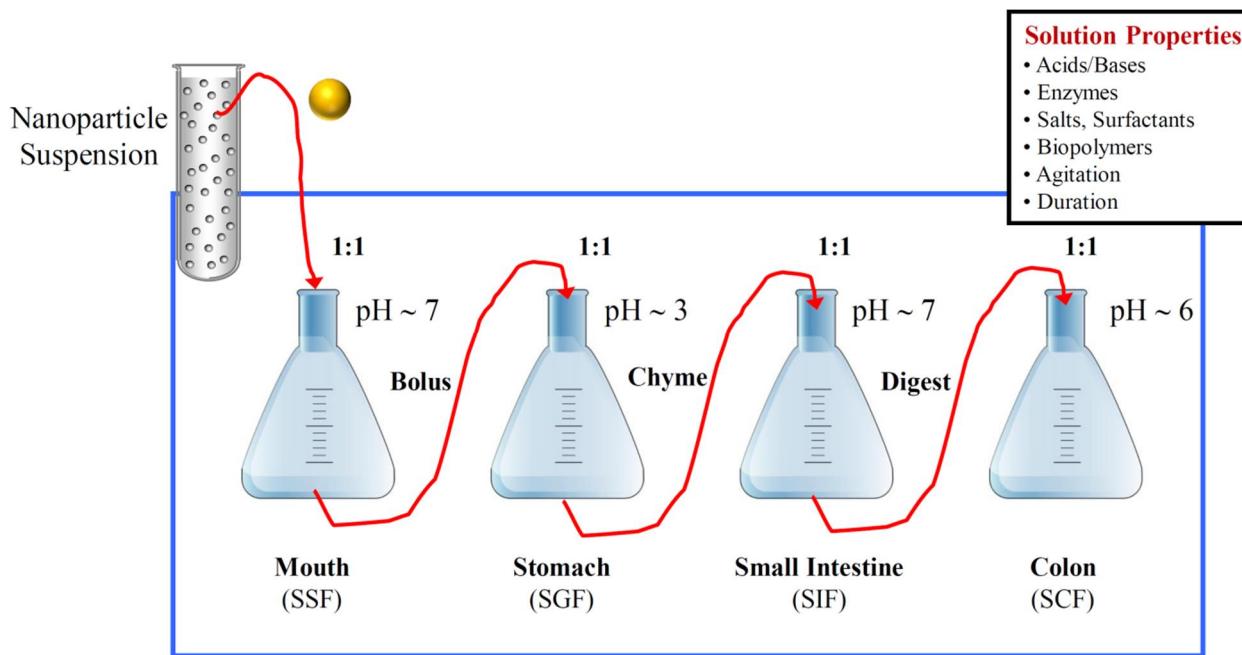


Figure 12: Schematic diagram of a multiple-step *in vitro* digestion model to simulate the gastrointestinal tract. A nanoparticle suspension is passed through a series of fluids that simulate the mouth, stomach, small intestine and colon, and changes in nanoparticle properties are recorded.

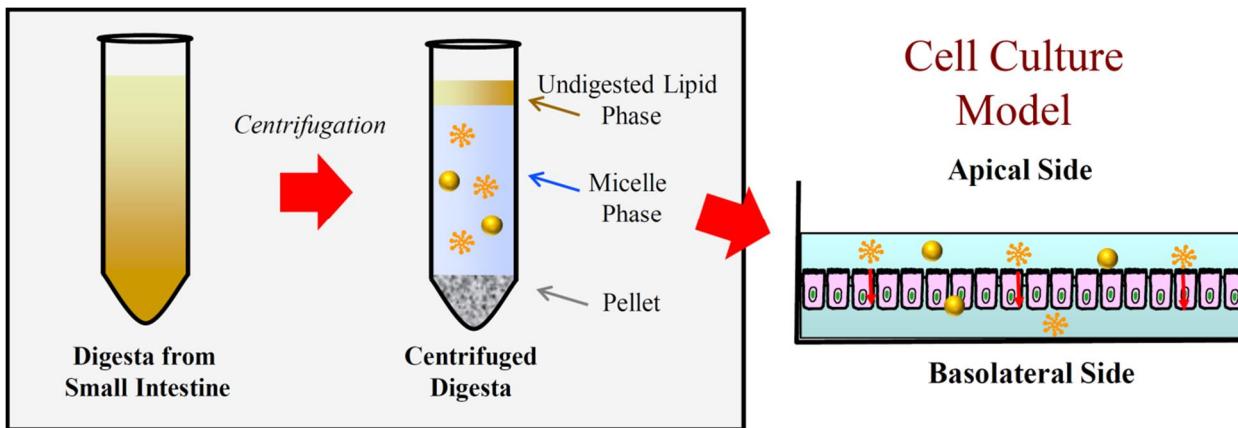


Figure 13: Schematic diagram of method used to measure the bioaccessibility and absorption of bioactive components or entire nanoparticles. Digesta resulting from the small intestine phase can be directly applied to a cell culture model, or it can be centrifuged first to collect the micelle phase and then placed on top of the cell culture model.

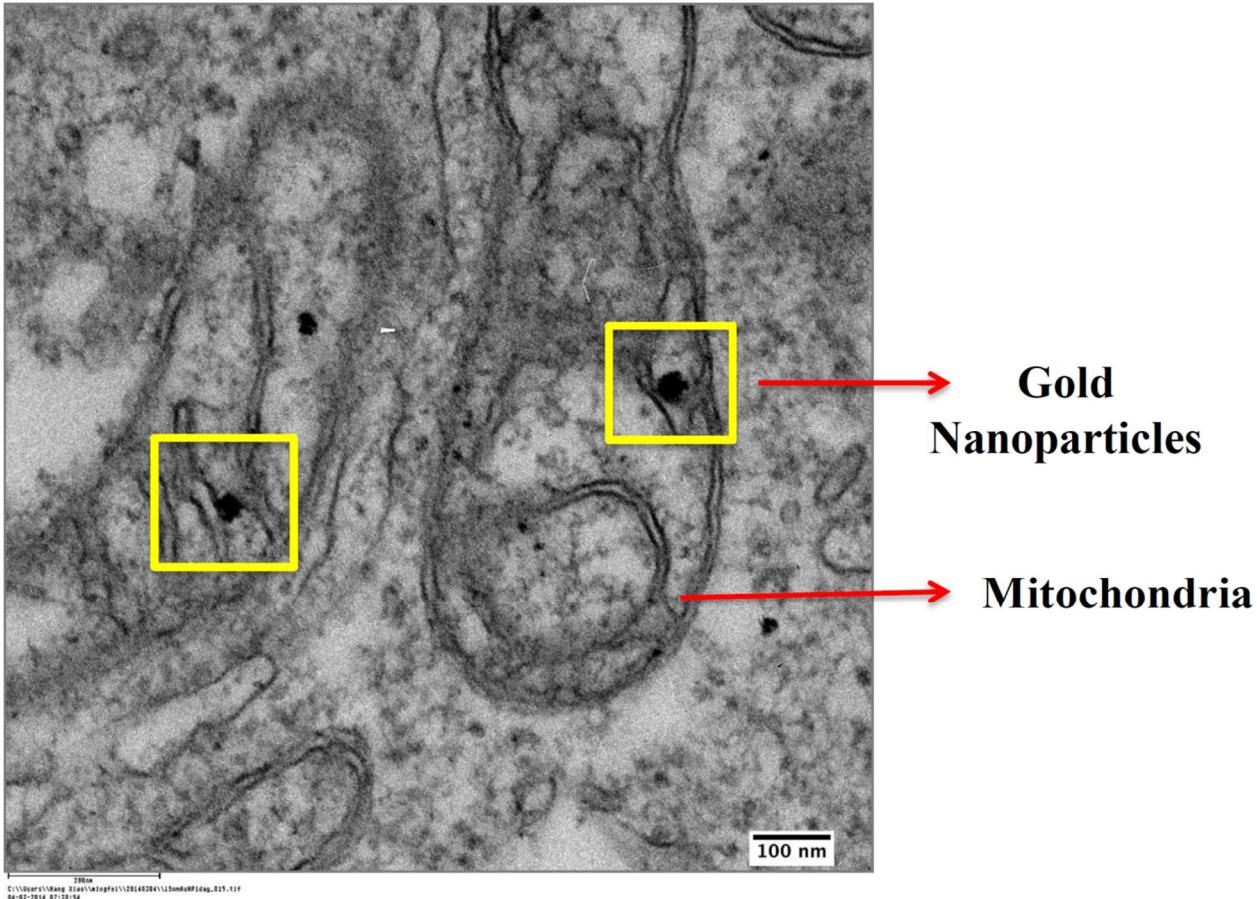
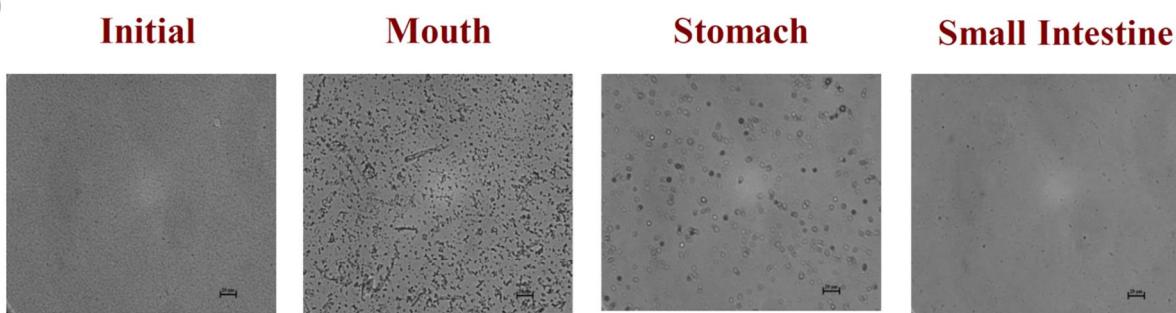
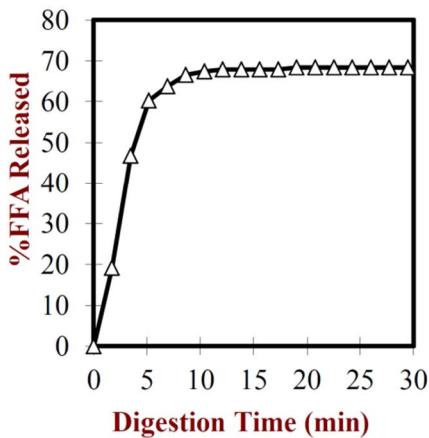


Figure 14: Transmission electron microscopy images of gold nanoparticles adsorbed by model epithelium cells (Caco 2 cells) highlighted by red box. This TEM image shows that some of the nanoparticles are located in the mitochondria (Kindly supplied by Olivia .Yao)

(A)



(B)



(C)

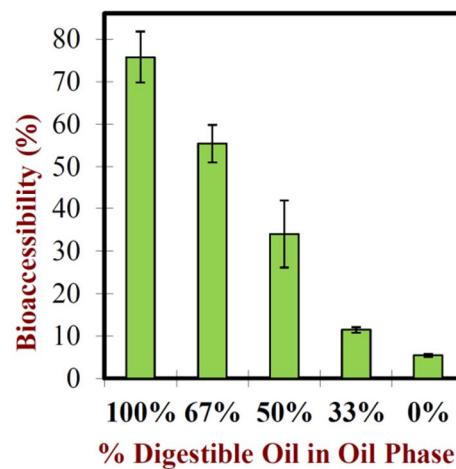


Figure 15: (A). Optical microscopy images of sucrose monopalmitate (SMP)-coated corn oil nanoparticles after passage through different regions of simulated gastrointestinal tract. (B). Free fatty acid release profile measured using a pH-stat for similar nanoemulsions under simulated intestinal conditions (Data provided by Jiajia Rao). (C) Influence of fraction of digestible oil on the bioaccessibility of β -carotene encapsulated within lipid nanoparticles containing different ratios of corn oil and lemon oil (Rao et al 2013).

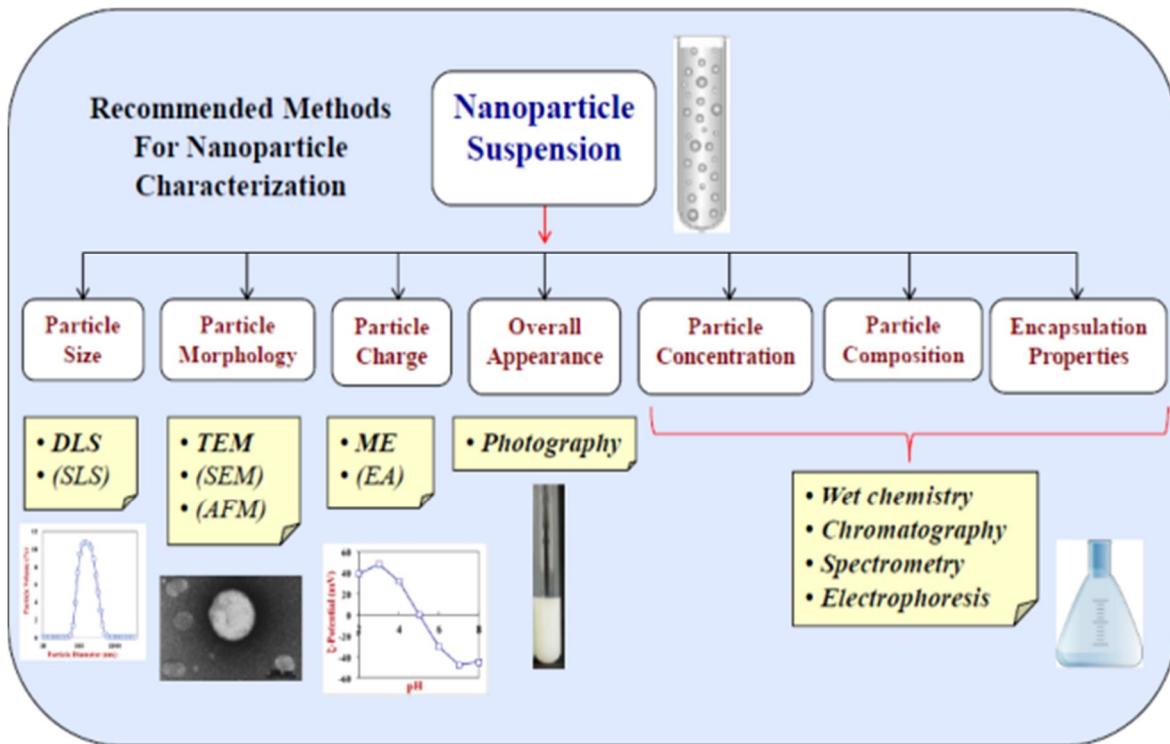


Figure 16: Summary of recommended methods for characterizing the properties of food-grade nanoparticles. The preferred methods are in bold, while alternative methods are in parenthesis. Key: ME = microelectrophoresis; EA = electroacoustics. Other abbreviations are given in text.