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REVIEW

A Review of Analytical Methods for the Identification and Characterization of Nano Delivery Systems in Food

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Detection and characterization of nano delivery systems is an essential part of understanding the benefits as well as the potential toxicity of these systems in food. This review gives a detailed description of food nano delivery systems based on lipids, proteins, and/or polysaccharides and investigates the current analytical techniques that can be used for the identification and characterization of these delivery systems in food products. The analytical approaches have been subdivided into three groups; separation techniques, imaging techniques, and characterization techniques. The principles of the techniques together with their advantages and drawbacks, and reported applications concerning nano delivery systems, or otherwise related compounds are discussed. The review shows that for a sufficient characterization, the nano delivery systems need to be separated from the food matrix, for which high-performance liquid chromatography or field flow fractionation are the most promising techniques. Subsequently, online photon correlation spectroscopy and mass spectrometry seem to be a convenient combination of techniques to characterize a wide variety of nano delivery systems.

KEYWORDS: Analytical methods; characterization techniques; food products; imaging techniques; nano delivery systems; nanoparticles; separation techniques

1. INTRODUCTION

The potential benefits for consumers and producers of the application of nanotechnology are widely recognized. Nanotechnology is a new and fast-emerging field that involves the manufacture, processing, and application of structures, devices, and systems by controlling shape and size at the nanometer scale. Nanoparticles (NPs) are defined as "a discrete entity with at least one dimension being 100 nm or less" (*1*). It is this small size in combination with the chemical composition and surface structure that gives a NP not also its unique features and its huge potential for applications but also its potential toxicological properties (*2*).

Products based on nanotechnology or containing NPs are already manufactured in the field of electronics, consumer products, and the pharmaceutical industry and are beginning to impact the food-associated industries (*3*). Here, nanotechnology tools are used in the entire food chain, for example, during cultivation (agriculture), industrial processing, and packaging of foods. In addition, nanotechnology is being used to enhance the nutritional aspects of foods by means of adding NPs (*4, 5*). A huge variety of NPs can be applied in food and agriculture, ranging from solid NPs (e.g., with metal cores, nanoscale products) with various forms and shapes to nano delivery systems (NDS). In the latter case, nutraceutical compounds (e.g., vitamins, probiotics, bioactive peptides, antioxidants, etc.) are incorporated, absorbed, or dispersed in small vesicles with nano diameters. This protects the nutraceutical compounds against degradation, improves the stability and solubility of the substance, and, therefore, increases the bioavailability and delivery to the target cells and tissues in the body (*6*). While larger delivery systems generally release encapsulated compounds more slowly and over longer time periods, reduction of the size of the delivery system introduces several bioadhesive improvement factors, including increased adhesive force and prolonged gastrointestinal transit time, leading to a higher bioavailability of active ingredients (*3*). In this way, NDSs can be used to enhance the controlled release of food ingredients at the right place and the right time. Until 2015, nano foods are believed to boost sales of tailor-made foods that deliver specific ingredients to target areas in the body (*7*).

The NDS may consist of a core composed of one or more substances surrounded by a wall or barrier. Various types of NDSs exist and are based on lipids, proteins, polysaccharides,

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polymeric networks, or combinations of these compounds. Polymer-based delivery systems have been developed extensively for the biomedical and pharmaceutical sectors to protect and transport bioactive compounds to target functions (*8*). In spite of successful elaboration of many synthetic polymers as delivery systems, these cannot be used for food applications, as food-grade polymers have to be utilized (*3*). Therefore, only delivery systems based on lipids, proteins, and/or polysaccharides are suitable for the application of nanoencapsulates into food.

Generally, there is good appreciation of the potential benefits of nanotechnology for the food industry and likely the consumer. However, not much is known on the safety aspects of the application of nanotechnology in food production and of the incorporation of NPs or NDSs in food products. One of the starting points for risk assessment, next to knowledge on toxicokinetic and toxicodynamic properties of the NPs, is the exposure assessment. For this, reliable information on the amount and type of NPs or NDSs is needed, and sophisticated techniques for determining NDS in food are highly desirable (*5*). It should be noted here that NDSs are lipid-, protein-, and polysaccharide-based systems and because the latter are being "generally recognized as safe" (GRAS), the NDS may be considered safe in food applications also or, at least, have a risk profile different from carbon-based or inorganic NPs.

In general, physicochemical properties of NPs to be considered are size and size distribution, surface area, shape, solubility and dissolution, reactivity, coagulation or aggregation state, chemical composition, and others (*9*−*13*). Functionalities of the NPs can change in different biological matrices (*12*), depending on compounds that are present in the matrix and thermodynamic conditions (*14*). In addition, NP interactions with the matrix can change as a result of dilution (*11*). In practice, this means that the sample processing itself, for example, means of isolation, concentration, and dilution, may influence the state of NPs present and thus the representativeness of the analysis.

With respect to NDS, the physicochemical properties are dependent on the type of delivery system. Therefore, a more detailed description of lipid-, protein-, and polysaccharide-based NDSs for food applications is presented in this review. Next, analytical methods capable of in situ detection and identification of NDS in food are reviewed, as well as separation techniques to isolate NPs or NDS from food prior to characterization. The analytical approaches that are evaluated in this review have been subdivided into three groups: imaging techniques, separation techniques, and characterization techniques.

2. NDSs

NDSs may use nanocapsules, nanocochleates, nanoballs, nanospheres, nanotubes, and other forms. As mentioned previously, these NDSs are composed of food-approved materials, and the compounds used are often lipids, proteins, polymers of saccharide or lactic acids, or combinations thereof. This chapter gives information about nanotechnology and NDS and the applications of lipid-, protein-, polysaccharide-, and poly(lactic) acid-based systems.

2.1. Nanotechnology and NDSs. Nanotechnology is an enabling technology that has the potential to revolutionize the food industry. Nanotechnology involves creating and manipulating organic and inorganic matter at the nanoscale. This can lead to far more precise and effective methods of manipulation of food polymers and polymeric assemblages to provide tailor-made improvements to food quality and food safety. The ability to design materials at the atomic or molecular level is likely to impact the food industry through the development of coatings, barriers, controlled release devices, and novel packaging materials. For instance, types of functional additives used in food, for which controlled release may be useful during either processing, consumption, or in the human body, are preservatives, antioxidants, redox agents (bleaching, maturing), acids, alkalizes, buffers, colors, flavors, sweeteners, nutrients, enzymes, and cross-linking agents. In this way, nanotechnology also promises to provide a means of altering and manipulating food products to more effectively and efficiently deliver nutrients, proteins, and antioxidants to precisely target nutritional and health benefits to a specific site in the human body or to specific cells to enhance their efficacy and bioavailability.

NDSs consist of solid hydrophobic nanospheres, nanotubes, or other forms composed of a blend of food-approved hydrophobic material (*15*−*17*). Various ingredients can be incorporated into or adsorbed to the NDS matrix, the surface of which can include a moisture-sensitive bioadhesive material, such as starch derivatives, natural polymers, natural gums, etc., making them capable of being bound to a biological membrane such as the oral cavity mucosa and being retained on that membrane for an extended period of time. NDSs can be localized and the target ingredient encapsulated within their structure to a particular region or a specific site, thereby improving and enhancing the bioavailability of ingredients that have poor bioavailability by themselves. Ingredients that have high water solubility, such as vitamin C, usually have low bioavailability. Enhancing the hydrophobicity of these ingredients by coupling them to a hydrophobic NDS enhances their bioavailability. Several applications of NDSs in food can be found in reports by ETC and Nanoforum (*18, 19*).

2.2. Lipid-Based Systems. Lipid-based NDSs are among the most promising encapsulation technologies employed in the rapidly developing field of nanotechnology. As compared to other encapsulation strategies, lipid-based nanoencapsulation systems have several advantages, including the possibility of being produced using natural ingredients on an industrial scale, targetability, and the ability to entrap compounds with different solubilities (*20, 21*). Lipid-based nanostructures that have been developed for drug delivery applications include lipid nanotubes, lipid nanospheres, and lipid NPs (*22*). The main lipid-based nanoencapsulation systems that can be used for the protection and delivery of foods and nutraceuticals are nanoliposomes, nanocochleates, and archaeosomes (*23*). Although their exploitation in food technology is yet to be explored, a recent study reported the use of nanostructured lipid carriers to disperse hydrophobic β-carotene in an aqueous phase, as a functional ingredient in beverages (*24*).

Liposomes are composed of one or more lipid and/or phospholipid bilayers and can contain other molecules such as proteins or polymers in their structure. Liposomes that contain only a single bilayer membrane are called small (<30 nm) or large (30−100 nm) unilamellar vesicles (*25*). These nanoliposomes have the significant advantage that they can incorporate and release two materials with different solubilities simultaneously. This means that these systems can accommodate water-soluble compounds together with lipid-soluble agents. Liposomes may release their contents into cells upon, for example, encountering specific cellular enzymes, due to pH or thermosensitivity, or after antigen-binding when an antibody is tagged. An example of liposome application in food is the entrapment of proteolytic enzymes for cheese production (*23, 26*). In this way, a cheese with good texture and flavor in half the normal time can be produced with considerably less enzyme required.

Liposomes have also been used to fortify dairy products with vitamins to increase their nutritional quality as well as to aid in digestion of constituents inherent to dairy products (*25*).

Archaesomes are liposomes made from one or more of the polar ether lipids extracted from the Archaeobacteria (*23*). As compared with liposomes (which are made from ester phospholipids), archaesomes are relatively more thermostable and more resistant to oxidation and chemical and enzymatic hydrolysis. They are also more resistant to low pH and bile salts that would be encountered in the gastrointestinal tract (*27*). This makes them ideal candidates to protect antioxidants during food processing.

Nanocochleates are stable lipid-based carriers comprised mainly of a negatively charged lipid and a divalent cation. They have a cigar-shaped multilayered structure consisting of a continuous, solid, lipid bilayer sheet rolled up in a spiral fashion with little or no aqueous space (*23*). In this way, hydrophobic, amphiphilic, negatively or positively charged molecules can be delivered. Because of their stability and nanometric size, nanocochleates have revealed great potential to deliver bioactive agents. They are resistant to degradation in the gastrointestinal tract, which makes them ideal candidates for oral delivery (*28*). At this moment, it has become feasible to use liposomes to deliver functional components such as nutraceuticals, antimicrobials, and flavors to foods (*24, 29*−*31*).

2.3. Protein-Based Systems. Proteins possess unique functional properties including their ability to form gels and emulsions, which allow them to be an ideal material for the encapsulation of bioactive compounds (*3*). Food proteins are widely used in formulated foods because they have high nutritional value and are GRAS. Protein hydrogels are the most convenient and widely used matrix in food applications. However, in the case of nonsolid and semisolid foods, it is essential to decrease the matrix size to allow their incorporation without affecting food sensory qualities (*32*). By decreasing the matrix size from micrometers to nanometers, new protein vehicles with improved delivery properties can be developed.

Protein-based NDSs are relatively easy to prepare in two different ways (*3*). The first one concerns the "top down" approach, where structures are generated by breaking up bulk materials; the second one concerns the "bottom up" approach, where structures are to be built from molecules capable of self-assembly. Self-assembly is a kind of aggregation whereby polymerization occurs toward the state of minimum free energy via noncovalent intermolecular forces, such as hydrogen bonding, electrostatic interactions, hydrophobic interactions, and Van der Waals interactions, as well as kinetically labile metal coordination (*33*). Linear assemblies such as rod- and tubelike structures are of particular interest, because they have unique properties with respect to applications (*34*).

During the preparation of protein NDS, no organic solvents are required and encapsulation is achieved under mild conditions, thereby minimizing destruction of sensitive nutraceutical compounds. For example, globular proteins such as whey proteins from milk have the ability to denature, dissociate, and aggregate under different conditions of pH, ionic strength, and temperature to form particles with sizes of 40 nm (*3*). Furthermore, partial hydrolysis of α-lactalbumine leads to the formation of stable tubular structures with diameters of only 20 nm (*35*). Another example of a protein-based NDS is gelatine NPs (*36*). The major benefit of these NPs is their simple and reproducible production combined with low costs and multiple modification opportunities offered by the matrix. However, as gelatine NDSs originate from animal slaughter material, the production of protein-based NDSs from plant proteins is preferred as plants are considered more safe (*37*).

Protein-based NDS are particularly interesting because various modifications allow them to form complexes with polysaccharides, lipids, or other biopolymers, and a wide variety of nutrients can be incorporated (*3*). These NPs can also conjugate nutrients via either primary amino groups or sulfhydryl groups (*38*). In general, controlled disassembly of the protein-based NPs results in controlled release of the encapsulated nutrient. It can be foreseen that food protein-based materials will play an important role in increasing the efficacy of functional foods over the next decade (*3*). However, at the present stage, greater fundamental understanding of protein−protein and protein−nutraceutical interactions at the molecular level and their impact on functional properties of proteins is still required to ensure the design of ideal nutraceutical carriers for use in the food industry.

2.4. Polysaccharide- and Poly(lactic) Acid-Based Systems. Polysaccharides are polymers of monosaccharides (carbohydrates) that are linked together by glycosidic bonds. These polymers are generally very large and often branched and are naturally occurring compounds in plants (e.g., pectin, guar gum, and insulin), animals (e.g., chitosan, chondroitin sulfate), algae (e.g., alginates), and microorganisms (e.g., dextran) (*39, 40*). They play diverse and important roles within the biology of life processes. The fact that these polymers are broken down by the colonic microflora to saccharides makes them interesting for NDSs, together with the fact that many polysaccharides are able to protect nutraceuticals from the hostile conditions of the stomach and small intestine. Hydrolysis of the glycosidic linkages on arrival in the colon triggers the release of the entrapped compound.

Up to now, chitosan is one of the most valued polysaccharides for drug delivery in biomedical sciences, most probably due to its permeability enhancer abilities (*41*). Chitosan is a polycationic polysaccharide derived from naturally occurring chitin by alkaline deacetylation (*40*). It is a copolymer of glucosamine and N-acetylated glucosamine, and it has favorable biological properties, such as nontoxicity, biocompatibility, and biodegradability. Another interesting polysaccharide for drug delivery purposes is pectin. Pectins are nonstarch, linear polysaccharides extracted from plant cell walls. They are predominantly polymers of mainly α-(1−4)-linked D-galacturonic acid residues interrupted by 1,2-linked L-rhamnose residues with an average molecular mass of about 50−180 kDa. Because of its water solubility, pectin needs structural modifications for its utility in targeted delivery. Polysaccharide-based encapsulation and targeted delivery systems are envisaged to have a potential for the development of food/nutraceutical formulations.

On the basis of their biodegradability and biocompatibility, homo- and copolymers derived from poly(lactic acid) and poly(glycolic acid) are being extensively utilized to prepare controlled release carriers for drugs and proteins. These aliphatic polyester polymers degrade by bulk hydrolysis of the ester bonds. Their therapeutic values have been increased in the form of micro- and NPs (e.g., NDS), which have shown clear advantages for parenteral and oral drug delivery. Most of the research work has been directed at poly(lactic acid), poly(glycolic acid), and poly(lactic-co-glycolic acid) copolymers. The success of these compounds for pharmaceutical applications has further led to the evaluation of aliphatic polyesters such as poly-ϵ-caprolactone (*42*).

3. IMAGING TECHNIQUES FOR NDSs

Depending on the matrix, microscopy can be used as a direct imaging technique for NDS, enabling not only quantitative analysis, the number of NDSs, but also qualitative information as the size, shape, and aggregation state of NDSs. Electron microscopy takes advantage of the wave nature of rapidly moving electrons. Where visible light has wavelengths from 400 to 700 nm, electrons accelerated to 10000 keV have a wavelength of 0.012 nm. Optical microscopes have their resolution limited by the diffraction of light to about 1000 diameters magnification. Electron microscopes are limited to magnifications of around 1000000 diameters, primarily because of spherical and chromatic aberrations. This chapter gives information about the methods that are used, or may be suitable, for the characterization of NDS.

3.1. Transmission Electron Microscopy (TEM). TEM is a nanoscale imaging technique that is capable of resolution on the order of 0.2 nm (*43, 44*). It is a technique whereby a beam of electrons is transmitted through a sample, and then, an image is formed, magnified, and directed to appear either on a fluorescent screen or on a layer of photographic film or to be detected by a sensor. The source radiation is generated using an electron gun. The resulting beam of electrons is focused into a tight, coherent beam by multiple electromagnetic lenses and apertures. The lens system is designed to eliminate stray electrons as well as to control and focus the electron beam. TEM is used heavily in both material science/metallurgy and biological sciences. In both cases, the specimens must be very thin and able to withstand the high vacuum present inside the instrument. For biological specimens, the maximum specimen thickness is roughly 1 μm. To withstand the instrument vacuum, biological specimens are typically held at liquid nitrogen temperatures after embedding them in vitreous ice or are fixated using a negative staining material or by plastic embedding.

There are a number of drawbacks to the TEM technique (*43, 44*). Many materials require extensive sample preparation to produce a sample thin enough to be electron transparent, which makes TEM analysis a relatively time-consuming process with a low throughput of samples. In addition, the structure of the sample may be changed during the preparation process. Also, the field of view is relatively small (3 mm diameter, less than 100 μm thick), raising the possibility that the region analyzed may not be characteristic of the whole sample. There is potential that the sample may be damaged by the electron beam, particularly in the case of biological materials. Moreover, TEM is an expensive technique.

TEM has already proved to be a suitable technique to image and characterize various kinds of NPs. For example, TEM has been applied to investigate milk protein-based nanotubes under different conditions (*45*), the shape of serum albumin NPs (*46*), and the fabrication of enzyme-incorporated peptide nanotubes (*47*). Furthermore, TEM has been used to control the size distribution and morphology of β-cyclodextrin nanospheres (*48*). TEM also provides valuable information on (nano)liposomal delivery systems since it yields a view of morphology and can resolve particles of varying sizes (*30, 49*−*51*).

3.2. Scanning Electron Microscopy (SEM). SEM is capable of producing high-resolution images of a sample surface (*52, 53*). Because of the manner in which the image is created, SEM images have a characteristic three-dimensional appearance and are useful for judging the surface structure of the sample. SEM is a technique where electrons are thermionically emitted from a tungsten or lanthanum hexaboride cathode and are accelerated toward an anode in a vacuum; alternatively, electrons can be emitted via field emission. The electron beam is collimated by electromagnetic condenser lenses, focused by an objective lens, and scanned across the surface of the sample by electromagnetic deflection coils. The primary imaging method is by collecting low-energy secondary electrons that are released by the surface of the sample. The secondary electrons are detected by a scintillation material that produces flashes of light from the electrons. The light flashes are then detected and amplified by a photomultiplier tube. Generally, TEM resolution is about an order of magnitude higher than SEM resolution; however, because the SEM image relies on surface processes rather than transmission, it is able to image bulky samples and has a much greater depth of view and so can produce images that are a good representation of the 3D structure of the sample.

An advantage of the application of SEM is that it has a large depth of field, which allows a large amount of the sample to be in focus at one time. With SEM, also, images of high resolution are produced, which means that closely spaced features can be examined at a high magnification. The combination of higher magnification, larger depth of focus, greater resolution, and ease of sample observation makes SEM one of the most heavily used methods in research areas today. However, SEM is also expensive and requires a high vacuum and sample conductivity (*54*). Sometimes, the presence of surfactants in the preparation may inhibit NP characterization via SEM due to the formation of a smooth camouflaging coating on the particle surfaces.

SEM has already been used to observe the morphology of polysaccharide NPs (*55*) and to study protein nanospheres for the encapsulation of essential oils to maximize the antimicrobial properties of the oils (*56*). Moreover, liposomal nanocapsules releasing their content were studied with SEM (*25*). All of these studies show the importance of SEM in characterizing NDS.

3.3. Atomic Force Microscopy (AFM). A more recently developed microscopic technique is AFM (*57, 58*). The high resolution ($\sim$0.1 nm) afforded by AFM has been utilized to directly view single atoms or molecules that have dimensions of a few nanometers. AFM relies on the raster scanning of a nanometer-sized sharp probe over a sample that has been immobilized onto a carefully selected surface, such as mica or glass, which is mounted onto a piezoelectric scanner. The tip is attached to a flexible cantilever. Deflection resulting from passage of the tip over the sample attributes is measured by a laser beam. The reflected laser beams are then detected at photodiode array detectors, which through a feedback mechanism, maintain the distance of the probe, amplitude of oscillation, or the cantilever deflection constant, depending on the scanning mode. The end result is a high-resolution three-dimensional profile of the surface under study. Different modes of AFM are available, including contact/repulsive mode (constant height, constant deflection, or tapping modes) and noncontact/attractive mode.

AFM was developed to overcome a basic drawback of scanning microscopy—that it can only image conducting or semiconducting surfaces. AFM has the advantage of imaging almost any type of surface, including polymers, ceramics, composites, glass, and biological samples. Unlike X-ray crystallography and electron microscopy, AFM allows biomolecules to be imaged not only under physiological conditions but also during biological processes. Because of the high signal-to-noise ratio, the detailed topological information is not restricted to crystalline specimens. Hence, single biomolecules without inherent symmetry can be directly monitored in their native environment. With AFM, native tissue can be directly observed without prior dehydration. The advantage of directly observing

biomolecular systems in their native environment opens the possibility to analyze their structural and functional properties at the submolecular level. Surface irregularities observed by SEM are absent on AFM inspection. With respect to the disadvantages of AFM, when the tip is in direct contact with the actual surface, it will run into difficulties should that surface be soft, sticky, or have loose particles floating on it. Nevertheless, AFM works on most materials and is heavily involved in a whole range of technologies ranging from biological, chemical, and electronics. Today, AFM images provide information on the surface structure of biomolecular systems, which is complementary to other established techniques such as light and electron microscopy, nuclear magnetic resonance (NMR), and X-ray crystallography.

AFM can be used for the structural characterization of proteins, polysaccharides, and liposomes (*59*). For example, AFM has been applied earlier to image the molecular structure of α-lactalbumin nanotubes with nanometer resolution in buffer and to probe their mechanical properties (*45*). AFM has also been utilized to study the morphology, size, stability, and dynamic processes of lipid nanocapsules (*60*−*62*).

4. SEPARATION TECHNIQUES FOR NDS

While some analytical techniques can be used for in situ detection of NDS, in most cases, this is not possible as the food matrix will interfere. For example, proteins in protein-containing food will interfere with the detection and identification of protein-based NDSs. Therefore, separation techniques are necessary to isolate the NDS from food prior to characterization. This chapter describes the most important separation techniques for isolating NDS. Because various detectors can be combined with the described separation techniques, the NDS can also be characterized to some extend.

4.1. Chromatography. *4.1.1. High-Performance Liquid Chromatography (HPLC).* HPLC is a form of liquid chromatography to separate, analyze, and quantify compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, high pressure pump, injector, analytical column (temperature controlled), and detector. Compounds are separated by injecting the sample mixture (carried by mobile phase) onto the column. The different components in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase (column material). Dependent on the type of stationary phase, compounds can be separated based on their charge (weak/strong cation or anion exchange chromatography), molecular mass [size exclusion chromatography (SEC)], hydrophobicity/polarity (reversed-phase HPLC, hydrophobic interaction chromatography), and specific characteristics (affinity chromatography). The most common detectors for HPLC are an ultraviolet−visible (UV−vis) light absorbance detector, a fluorescence detector, an electrochemical detector, and a diffractometer.

HPLC has been used in food analysis for measuring numerous compounds, for example, carbohydrates, vitamins, additives, mycotoxins, amino acids, proteins, tryglycerides in fats and oils, lipids, chiral compounds, and pigments. It is a straightforward, robust, and reproducible technique, and the price of an HPLC instrument is modest. Sensitive selective detectors are available to utilize with HPLC depending on the compound to be analyzed. Another advantage of HPLC is that both the stationary and the mobile phases can be selected to achieve the necessary separation of sample components. Furthermore, separated compounds can be collected as they elute from the column. Moreover, the rather new monolithic HPLC columns provide higher efficiency and better permeability than the conventional HPLC columns, allowing a higher flow rate and therefore reducing total analysis time (*63*). One of the most important trends is the miniaturization of the analytical systems. Microparticles and microcolumns have been developed for HPLC, leading to a technique called ultraperformance LC (UPLC), providing significant advantages concerning selectivity, sensitivity, and speed (*64*). Further miniaturization leads to nano-LC, a technique often used in proteomic research and having an even better performance than UPLC (*65*), while two-dimensional LC makes HPLC an even more valuable and powerful analytical tool (*66*).

Because size and/or charge are typical characteristics of NDS, SEC and/or ion exchange chromatography (IEC) are the most suitable types of liquid chromatography for the separation of NDSs from the food matrix. In the case of SEC, compounds are separated on the basis of size; larger compounds (e.g., NDSs) elute faster than smaller ones. By using calibration mass standards, it is possible to estimate the molecular mass of a compound. However, caution should be taken as the elution could be affected by the shape of the compound. In addition, the elution position will change if the compound has any tendency to interact with the column matrix.

In the case of IEC, compounds are separated on the basis of charge; low charged compounds elute faster than highly charged ones. During SEC or IEC, the elution of protein NDS can be monitored via UV/vis detection and/or using intrinsic fluorescence detection (*67*). Liposomal delivery systems can be detected during SEC elution by collecting fractions and analyzing them by photon correlation spectroscopy (PCS) (*68*). By additional analyses of the collected fractions with an enzymatic phosphatidylcholine assay, the lipid content can be determined as well. This gives a more detailed and reliable insight into the particle size distribution of liposomes than PCS alone. SEC coupled to refractive index (RI) and multiangle light scattering (MALS) detectors has been used to characterize polysaccharides (*69, 70*). An alternative is SEC followed by detection by online electrospray ionization−mass spectrometry (ESI-MS) or off-line matrix-assisted laser desorption ionization−time-of-flight−MS (MALDI-TOF-MS) (*71, 72*). Here, the composition, size, and sequence of the repeating unit of polysaccharides are determined.

4.1.2. Field Flow Fractionation (FFF). FFF is a flow-assisted separation technique for the separation of analytes in a 10^{15} molar mass range, from macromolecules such as proteins (nanometer range) to micrometer-sized particles such as whole cells (*54, 73*). Very broad ranges of molecular sizes can be separated in one single run. As in HPLC, FFF starts with the injection of a sample into a stream flowing through a thin, empty flow chamber or channel. The dimensions of this narrow ribbonlike channel are typically 50 cm in length and 2 cm in width, whereas the channel height varies between 50 and 500 μm. Unlike HPLC, FFF has no stationary phase excluding any interfering interactions between the analytes and the stationary phase (*73*). From the inlet, a carrier liquid (mobile phase) is pumped through the channel, establishing a parabolic flow profile as in a capillary tube, propelling the samples toward the outlet. Separation is achieved by the interaction of sample components with an externally generated field, applied perpendicularly to the direction of the mobile phase flow. Thermal gradients, hydraulic, sedimentation, and electrical forces are utilized in FFF to create this transversal field force. The selectivity originates from the differential distribution of various

analytes across the parabolic flow profile. Analytes move with the perpendicular force to the side of the channel, but small analytes diffuse back to the center of the channel more easily. Because the mobile phase flow is higher at the center of the channel, smaller analytes elute earlier than larger ones. At the end of the channel, the analytes are flushed out into a detector (e.g., based on UV−vis absorbance or laser scattering) or a collection device for further characterization.

FFF has significant advantages over other bioseparation techniques in terms of high biocompatibility, "soft" fractionation mechanism, reduction of sample carry-over, and simple sterility issues. The open-channel configuration also makes FFF advantageous for continuous fractionation of bioanalytes on a preparative scale. The total separation times typically range from a few minutes up to 30 min. A limitation of the FFF system is that the channel is easily overloaded. Such undesired effects are usually recognized by peak skewness, peak broadening, and loss of resolution. If this is the case, samples should be diluted. As a consequence of this, the detection of trace amounts of compounds is limited by the sensitivity of an appropriate detector system. In comparison to SEC, FFF offers superior resolution for samples with a molecular mass higher than 100 kDa but lower resolution in the range below 50 kDa (*54*). Furthermore, FFF separation enables the characterization of insoluble analytes aside from soluble specimens.

FFF has been used to determine the mass of proteins and size of colloidal carriers, including liposomes, injectable emulsions, and particles (*62*). Flow FFF, which uses a mobile phase crossflow as the applied field, also called asymmetrical FFF, has been the most widely applied FFF technique for protein fractionation (*73*) and fractionation of polysaccharides like chitosan (*74*). Flow FFF is particularly appealing for the separation of intact ultralarge proteins and protein complexes, as there is no interaction possible with a stationary phase and the absence of organic modifiers in the mobile phase (as in HPLC) precludes degradation (*75, 76*). With flow FFF, it is possible to separate and quantitate protein aggregates. The different retention times for various aggregate peaks usually indicate the size of the aggregates since the retention times can be related to the diffusion coefficients. In a similar way, a flow FFF method in combination with MALS demonstrated the ability to comprehensively characterize polymeric gelatine bulk material as well as drug-loaded and unloaded NPs in terms of size, size distribution, molecular mass, and loading efficiency (*77*). Because FFF can also be coupled to MS for protein characterization, this technique can also be a promising method for detecting and characterizing NDSs composed of (glyco)-proteins (*73*). Comparable with SEC in the previous section, FFF combined with MALS and differential RI can be used to characterize polysaccharides (*78*). This provides a promising technique for the characterization of polysaccharide NDSs. With respect to the characterization of lipid NDS, FFF in combination with MALS detection can be applied (*79*). Finally, electric FFF appears to be a powerful technique to separate NDS mixtures of different surface charges and particle sizes (*80*).

4.1.3. Hydrodynamic Chromatography (HDC). The separation principle of HDC relies on the size-dependent exclusion from the wall in a microchannel in which a pressure-driven flow is applied (*81*). In narrow conduits (effective size <1 μm) with a laminar flow, larger molecules or particles (size range from 0.002 to 0.2 of the conduit size) are transported faster than smaller ones as they cannot fully access slow-flow regions near the conduit walls (*62*). In HDC, this is used for analytical separation in applications similar to traditional SEC. Classical HDC is performed in columns packed with nonporous particles giving a relatively small efficiency especially for small packing particle sizes. Nowadays, the separation effect occurs in packed columns, open microcapillaries, or flat microchannels. The dynamic range of packed column HDC is from molecular size up to particles of greater than 1 μm. HDC can be combined with several detection systems. The most popular one are based on UV absorbance and laser scattering (*81, 82*). Eventually, the chromatogram of retention time vs response is converted to a particle size distribution using a combination of calibration procedures and peak fitting models (*83*).

HDC is suitable for size characterization of synthetic polymers, biopolymers, and particles in solution or suspension (*83*). In particular, HDC in combination with UV detection was found to be an excellent tool for the determination of size and average size distribution of lipid nanocapsules (*62, 84*). With the HDC setup, reproducible chromatograms at different flow velocities can be obtained (*81*). The development of an on-chip HDC system shows some possible advantages over conventional techniques, such as SEC and FFF. These include fast analysis, high efficiency, reduced solvent consumption, and easy temperature control. However, the noise level of the detection is high and unstable during the experiments. A drawback of HDC in general is clogging of the column (*85*).

4.2. Electrophoresis. *4.2.1. Capillary Electrophoresis (CE).* CE can be used to analyze and characterize a wide variety of analytes ranging from simple inorganic ions, small organic molecules, peptides, proteins, nucleic acids to viruses, microbes, and particles (*86*). CE comprises a family of electrokinetic separation techniques that separate compounds based upon differences in electrophoretic mobility. This mobility can be controlled by the charge/mass ratio of the analyte, isoelectric point, molecular size, or hydrophobicity depending on the separation conditions. The most employed modes of CE include capillary zone electrophoresis, capillary isoelectric focusing, capillary gel electrophoresis, and capillary electrochromatography. The main components in CE instrumentation are a sample vial, source and destination vials, a capillary (internal diameter, 10−100 μm), electrodes, a high-voltage power supply, and a detector. The source vial, destination vial, and capillary are filled with an electrolyte such as an aqueous buffer solution. The sample is normally introduced by placing the capillary inlet into the sample vial and applying pressure. After returning the capillary inlet into the source vial, the migration of analytes is initiated by applying an electric field between the source and the destination vials. This electric field is supplied to the electrodes by the high-voltage power supply. All ions, positive or negative, are pulled through the capillary in the same direction by electro-osmotic flow. The analytes separate as they migrate due to their electrophoretic mobility and are detected near the outlet end of the capillary. Separated chemical compounds appear as peaks with different retention times in an electropherogram. Typical CE detectors are based on UV/vis absorbance, fluorescence, conductivity, or MS (*87*).

In general, CE is simple, rapid, and low cost, because it needs neither laborious treatment of the samples nor long times for analysis (*88*). Moreover, high separation efficiency can be achieved. The main disadvantage of CE is its lower reproducibility when compared to other (chromatographic) methods. Furthermore, although the sample volume usually consumed per CE analysis is a few nanoliters, the sensitivity in terms of concentration is not very high, which precludes the use of CE for the determination of trace compounds. However, extremely sensitive detection systems like a laser-induced fluorescence and

MS detector enhance the sensitivity (*87*). The use of multicapillary arrays and chip-based separations is very helpful to overcome throughput limitations (*89*).

CE has been employed to a large extent to examine liposomes and characterize them in terms of size, surface charge, rigidity, permeability, and stability (*90*−*92*). CE appears to also be applicable to separate and characterize polysaccharide NDSs as CE has been used in earlier studies for the separation and analysis of chitosan varieties (*93*). This is supported by investigations on lipopolysaccharides via CE-MS (*94*). With respect to the analysis of (glycol)proteins and protein complexes, CE in combination with MS or laser-induced fluorescence proved to be suitable methods (*95*−*97*). It must be noted here that coating of the capillaries supports efficient and reproducible analysis of proteins by CE (*98*). These studies are also promising for separating and analyzing protein NDSs with CE.

4.2.2. Gel Electrophoresis. Gel electrophoresis is a technique for separating a mixture of biomolecules (DNA, RNA, and proteins) through a stationary material (gel) in an electrical field (*99*). It is usually performed for analytical purposes but can also be used as a preparative technique to purify molecules prior to characterization techniques such as MS. The gel is usually composed of different sized mesh networks of polyacrylamide wherein smaller molecules migrate faster than larger molecules. Electrophoresis refers to the electromotive force (electric current) that is used to push or pull the molecules through the gel matrix. After the electrophoresis runs, the migrated molecules in the gel can be stained for visualization. The resulting band intensities are indicators of the concentration. One of the profits of gel electrophoresis is the fact that up to tens of samples can be analyzed on one gel at once. However, the total analysis time takes several hours. This depends on the electrophoresis apparatus, the applied gels, and the running conditions. For example, gel electrophoresis can be conducted on precast gels, saving time, and the trouble and hazard of working with acrylamide. On the other hand, an advantage of making one's own gels is the much lower costs.

In native or nondenaturing polyacrylamide gel electrophoresis (native-PAGE), the mobility of biological molecules in the gel depends on its charge and hydrodynamic size. This means that molecules with the same molecular mass but with a different hydrodynamic size will run differently on the gel. The mobility of compact conformations will be higher than that of more extended structures. If native-PAGE is carried out near neutral pH to avoid acid or alkaline denaturation, then it can be used to study conformation, the binding of compounds, and self-association or aggregation (*100, 101*). In this respect, it can be useful for the characterization of protein NDS. An advantage of native gels is that it is possible to recover biological molecules like proteins in their native state after the separation. Native-PAGE cannot be used to measure the molecular mass of biological molecules as the mobility in the gel is affected by both charge and hydrodynamic size. However, in the presence of detergent sodium dodecyl sulfate (SDS), the biological molecules have a uniform negative charge, and for proteins, a linear polypeptide chain is formed. Therefore, SDS-PAGE is a technique that allows the determination of the molecular mass of a molecule. Here, molecular mass standards are used to generate a curve correlating molecular mass and migration in the gel, from which the molecular mass of the unknown sample can be determined.

The simultaneous ability of SDS-PAGE to determine the size and amount of a biological molecule is also useful to determine protein aggregates like protein NDSs (*101*). Under nonreducing conditions, this technique is capable of revealing covalent protein aggregates whereas under reducing conditions protein aggregates not based on disulfide bonds can be revealed. In general, noncovalent protein aggregates cannot be analyzed by SDS-PAGE as SDS disrupts the interactions between the proteins that form aggregates. Furthermore, PAGE is not suitable for analyzing insoluble protein aggregates as they will remain on top of the gel and will not migrate into the gel.

5. CHARACTERIZATION TECHNIQUES FOR NDS

Separation techniques are primarily used to isolate NDSs from the matrix and have only a limited capability for NDS characterization. For a more extended characterization, for example, physical and chemical characterization techniques are required. This chapter describes a number of important characterization techniques that are already used or can be used for the characterizing NDSs. While some of these may be used as stand-alone techniques, others can be used in combination with chromatographic techniques mentioned in the previous chapter.

5.1. MS. MS is a powerful analytical technique for measuring the mass-to-charge ratio of ions (*102*). In general, MS is applied to elucidate the chemical composition of a sample by generating a mass spectrum representing the masses of the sample components. This is achieved by first ionizing the sample, separating the ions of differing masses, and recording their relative abundance by measuring intensities of the ion flux. In recent years, enormous progress has been made in the application of MS to studies of biomacromolecules. This has been possible because of the development of new ionization techniques, for example, MALDI, ESI, and more recently desorption electrospray ionization (DESI).

5.1.1. MALDI. MALDI was introduced in 1988 as a method of transferring large, labile molecules into the gas phase as intact ions (*103*−*105*). For the analysis of proteins or peptides, the technique involves mixing the analyte with a large molar excess of a UV-absorbing matrix compound, usually a weak organic acid. After drying and crystallization, this mixture is placed on a vacuum probe and inserted into the MALDI-MS for laser desorption analysis. The matrix strongly adsorbs the laser light at a wavelength at which the analyte is only weakly absorbing, reduces intermolecular contacts beyond analyte−matrix interactions thereby reducing desorption energy, and acts as a protonating (or deprotonating) agent and is therefore essential in the ion formation process. MALDI generally leads to the formation of single charged ions, and the previously mentioned effects result in high ion yields of the intact analyte, giving rise to subpicomole sensitivity.

The pulsed laser used for MALDI makes it an ideal ionization technique for TOF-MS since there is a precisely defined time of ion generation. The ionized biomolecules are accelerated electrostatically to a defined kinetic energy, and the flight time is determined by a detector positioned at the end of the field-free region. At a fixed kinetic energy, small ions travel at higher speed than large ions, that is, they have a shorter flight time. Typical TOF-MS features are acquisition of complete mass range spectra in microseconds, in principle no upper mass limit, a high sensitivity due to a high ion transmission, and improved mass resolution by the use of an ion mirror (reflectron).

MALDI-TOF MS can be used for the detection and characterization of proteins, peptides, oligosaccharides, and oligonucleotides with molecular masses in the range of 400 up to 200000 Da and, thus, also for NDSs. Although MALDI-MS has been used extensively to provide molecular mass and

structural and compositional information of synthetic polymers, a limitation of the technique is that it fails to provide correct molecular mass values for mixtures of proteins and peptides. An experimental procedure to overcome this limitation involves combining MALDI with chromatography. SEC techniques like gel permeation chromatography have the advantage that they can separate complex samples on the basis of molecular size, but they have the disadvantage of low mass resolution. MALDI has the advantage of high mass resolution and high mass accuracy with the disadvantage of sample discrimination and inaccurate average molecular mass for mixed samples.

Three common methods are used to combine MALDI with SEC. In the first method, individual SEC fractions are collected and analyzed by MALDI. While this method is tedious because many samples have to be generated, it does provide absolute mass calibration points for the SEC chromatogram based on the sample itself instead of external standards. In the second method, SEC fractions are collected continuously by spraying it or combining it with an appropriate target matrix followed by MALDI (*106, 107*). The third, and most efficient, method would be online coupling of MALDI with LC (*108−110*), which has been studied by various research groups, in most cases with peptides as the target analytes. In this method, the effluent from the HPLC column is mixed with the solution of the matrix in a T-piece, the third leg of which is connected to the MALDI ionization chamber by a capillary tube with a stainless steel frit at the end. The HPLC effluent crystallizes together with the matrix on the MS side of the frit, and a laser beam is used to produce the required effect by MALDI on the crystallized effluent. The idea is to continuously regenerate the interface through combined actions of solvent flushing and laser ablation. Another technique used for peptide identification in combination with MALDI-TOF is PMF.

While MALDI has good potential for the characterization of NDSs and other organic NPs, only limited information is available describing the actual characterization of NDSs using MALDI. In fact, many applications deal with the opposite, the use of NPs as probes for certain analytes in MALDI analysis (*111−113*). PEG is a biocompatible polymer that has been studied using MALDI and that is widely used for preparing bioconjugates with biologically relevant molecules, including peptides, proteins, and low molecular weight drugs. These types of conjugates are under development as NDSa and for other medical applications. MALDI is used as a technique for the characterization of reactive PEG derivatives and their conjugates (*114, 115*). Studies are being conducted to tune the dimensions of polylactides, biodegradable polymers used as NDSs, by choosing different block copolymers in the synthesis. Structural characterization of the synthesis products is carried out using MALDI-TOF MS (*116*).

5.1.2. ESI. ESI is commonly used as the ionization technique in the coupling of liquid chromatographic separation with mass spectrometric detection (*117−119*). The basis behind ESI is the formation of charged molecules in a solution and then transferring them into the gas phase under atmospheric pressure (*120, 121*). ESI is considered a soft ionization technique as it does not induce fragmentation in the ionization process. The ability to form multiple charged ions is of great value for the measurement of large molecules with relatively simple equipment (*122*).

The basic process in ESI is that electrolytes in solution are transferred into the gas phase by means of an electric field and an electric potential applied to a tip of a capillary. When a positive potential is applied to the tip of the capillary, positively charged molecules will accumulate at the meniscus of the solvent. When the positive charge increases, the meniscus is destabilized by the electric field, and a spray is formed. By evaporation of the solvent, the droplets undergo further reduction in size, and finally, molecules are transported into the mass spectrometer. With ESI, positive as well as negative ions can be created. Proteins and peptides are normally analyzed as positive ions. Common mass analyzers combined with ESI are a triple quadrupole (QqQ), a TOF, a quadrupole time-of-flight (Q-TOF) combination, and an ion trap (ITD).

While LC enables the separation of proteins and peptides in complex samples, a direct coupling of LC with ESI-MS enables the identification of these biomolecules. For this, LC-MS analysis primarily reversed-phase chromatographic columns are used (*123−125*) in combination with an organic modifier and an organic acid as an ion-pairing agent (*126, 127*). Mass spectra can be complex due to the limited LC separation and the generation of multiple charged ions, and deconvolution software is required to calculate molecular mass.

Peptide mass fingerprinting (PMF) is used in combination with ESI-MS for protein identification and characterization (*128−135*). In this method, the unknown proteins are first digested into smaller peptides, whose absolute masses can be accurately measured with ESI-TOF or MALDI-TOF (*136*). These masses are then in silico compared to a database containing known protein sequences and statistically analyzed to find the best match. Typical for the PMF-based protein identification is the requirement for an isolated protein. Mixtures exceeding a number of 2−3 proteins typically require the additional use of MS/MS-based protein identification to achieve sufficient specificity of identification (*137−139*).

As with MALDI, the potential of ESI-MS for the identification and characterization of peptides suggests that this technique will also be suitable for the characterization of biodegradable polymers used as NDSs or building blocks thereof. Again, only a few examples can be found in the literature, and one example of the ESI-MS characterization of NDS building blocks can be found in a study of the self-assembly of PEG-oligolactates and PEG-oligocaprolactones with monodisperse hydrophobic blocks (*140, 141*). The same authors used ESI-MS to study the mechanism of the chemical and enzymatic degradation of PEG-oligocaprolactones, micelles that are used in the biomedical field as microspheres and nanospheres that can be used for the controlled delivery of several types of drugs (*42, 142*).

5.1.3. DESI. DESI is a relatively new ionization method for MS that allows direct sampling of analytes from surfaces under ambient conditions. Using a nebulizer, electrosprayed charged droplets and ions of solvent are directed onto the surface to be analyzed. The impact of the charged particles on the surface produces gaseous ions of material originally present on the surface, collected in the inlet of a mass spectrometer that is positioned closely to the surface (*143−145*). The resulting mass spectra are similar to normal ESI mass spectra in that they show mainly singly or multiply charged molecular ions of the analytes. The DESI phenomenon is observed in both conductive and insulator surfaces and for compounds ranging from nonpolar small molecules to polar compounds such as peptides and proteins. Changes in the solvent that is sprayed can be used to selectively ionize particular compounds. Typically, ionizable molecules that are amenable to conventional ESI-MS are also amenable to analysis by DESI-MS. As a surface sampling technique, DESI-MS can be easily used for the analysis of thin-layer chromatography (TLC) plates. Applications include the analysis of dyes, small drug molecules, alkaloids, and pharmaceutical products (*146−149*). A drawback is that DESI is only

applicable for solid surfaces, and as it analyzes only the surface of the sample, the results may not be representative for the whole sample. While there are examples of DESI analysis of peptides and proteins, no information was found about the analysis of polysaccharides and liposomes with DESI.

In the past few years, proteins, peptides, and tryptic digests deposited on planar media have also been analyzed frequently using surface sampling with DESI-MS (*150*−*154*). Peptides from digests can be identified using DESI-MS in the full-scan mass spectra mode or DESI in combination with data-dependent tandem mass spectra as is in PMF. It is observed that polar peptides give a poor DESI-MS response, probably due to their stronger surface interactions with the hydrophilic substrate on TLC plates. In addition, signal suppression may occur if chromatographic separation is limited. Nevertheless, surface sampling using DESI-MS shows a great potential for the analyses of proteins and peptides separated by TLC or HP-TLC and may also be used for the characterization of NDS.

5.1.4. Ion Mobility Spectrometry (IMS). With the development of new ionization techniques as ESI, MALDI, and DESI, IMS has moved from a technique limited to volatile analytes in vapor phase samples to nonvolatile analytes in other matrices (*155*−*157*). IMS is based on the differential migration of gas phase ions through a buffer gas in a homogeneous electric field and is thus capable of separating ions on their size/charge ratio as well as their interactions with the buffer gas. As such, it has become a powerful analytical tool for investigating molecular structure and separating complex samples as found in applications of proteomics, glycomics, and metabolomics (*158*−*160*). The coupling of IMS with MS is often referred to as ion mobility-mass spectrometry (IMMS). Sample introduction of liquid or solid samples is most often achieved using ESI (*161*) and MALDI (*162, 163*), respectively.

The workhorse of IMMS is an IMS coupled to a TOF-MS (*164*). By coupling of IMS to a TOF-MS analyzer, ions are pulsed into the MS, resulting in the final ability to separate ions in two dimensions, by their mobility and *m*/*z* ratio. IMMS allows rapid separation of components by size, shape, and mass, where ionic species of different charge states can be isolated in different ion arrival distributions. Therefore, this opens opportunities for the analysis of NDS since a unique feature of IMMS spectra is that they often contain a mass mobility correlation for classes of ions. In IMMS spectra, the drift time (or collision cross-section) is plotted against mass (or mass/charge ratio), and trend lines can be observed (*165, 166*). In general, compounds of the same class and similar structures will follow the same trend line. However, for classes of compounds where the structures are dissimilar, large variations may occur. Thus, in relation to the characterization of NDS systems, IMMS allows the identification of the type of NDS system, lipid-based, protein-based, or polysaccharide-based.

The potential of IMS for separating shapes of heterogeneous macromolecules allows one to distinguish between isomeric peptides, disaccharide isomers, and isobaric di- and trisaccharides (*167*). In general, the analysis of carbohydrates, and thus the characterization of polysaccharide-based NDS with IMMS, is promising because of the large number of isomers and the difficulty of distinguishing those isomers by MS alone. LC separation of sugars prior to IMS will further aid in identification (*168*). The need for high-throughput methods for proteomics makes IMMS an ideal analytical tool for proteins and peptides and thus for protein-based NDSs. Tryptic digests of proteins are introduced via liquid chromatography (*169*), directly infused into the IMMS (*170*), or introduced via desorption ESI (*158, 171*).

Apart from saccharides and proteins, IMMS is also used for the analysis of lipids. Because lipids have a lower density than proteins and saccharides, they produce a trend line separate from those and other potentially interfering biomolecules (*172*). NDSs are used as delivery vehicles for drugs, and changes in ion density between the lipid-NDS and the drug-loaded lipid-NDS can be observed with IMMS (*173*). Therefore, IMMS may not only allow the identification of the type of NDS (lipid-, protein-, or polysaccharide based), but also determine whether the NDS is loaded or not.

5.2. PCS. Nowadays, PCS is the standard method for rapidly determining the particle diameter and size distribution of NPs (*62, 174*). PCS is also referred to as dynamic light scattering (DLS) and quasi-elastic light scattering. The pace of the movement of particles in water is inversely proportional to particle size and can be detected by analyzing the time dependency of the light intensity fluctuations scattered from the particles when they are illuminated with a laser beam. With PCS, one measures the time dependence of the light scattered from a very small region of solution, over a time range from a 10th of a microsecond to milliseconds. These fluctuations in the intensity of the scattered light are related to the rate of diffusion of particles in and out of the region being studied (Brownian motion), and the data can be analyzed to directly give the diffusion coefficients of the particles doing the scattering. Traditionally, rather than presenting the data in terms of diffusion coefficients, the data are processed to give the "size" of the particles (radius or diameter), which is based on a theoretical relationship between the Brownian motion and the size of spherical particles.

Because of its noninvasive and nondestructive performance, PCS evades artifacts (*77*). Dissolved and nondissolved matter can be sized within minutes in a reproducible way. Moreover, PCS is accurate and simple to perform. The major obstacle to achieve veritable results concerns the fact that the derived sizes are influenced by the presence of dust or agglomerated fractions present in the sample. Furthermore, a number of assumptions inherent in data analysis also affect particle distributions. Many of these issues were addressed in a study, in which SEC was coupled with both static and DLS, and PCS was found to be only applicable for high molecular weight polymers with a higher RI increment (*175*). However, PCS is to be considered the preferred choice for NP sizing, provided that size distributions are narrow.

PCS provides a fast and adequate evaluation of the size of nanoliposomes as NDSs. This has been proven in various medical studies (*51, 92*) and food studies (*31, 176*). In these latter studies, polypeptide antimicrobials encapsulated in liposomes may offer a potential solution to protect antimicrobials and enhance their efficacy and stability in food applications. With respect to protein-based NDS, for example, gelatine NPs have been characterized by sizing via PCS (*36, 77*). Finally, also, the size, size distribution, and stability of delivery systems based on polysaccharides have been elucidated by PCS (*177, 178*). The results from one of these studies may be used to promote the value-added utilization of chitosan and to improve the efficacy and safety of nutraceuticals and functional foods (*178*).

5.3. Analytical Ultracentrifugation (AU). AU is an extremely versatile and powerful tool for the characterization of biological macromolecules and the interactions between them (*179, 180*). AU is applicable over a wide range of molecular masses from approximately 2.5 kDa up to 1.5 MDa. The AU instrument spins a sample under vacuum at a controlled speed and temperature, while at set times the concentration

distribution is recorded. Monitoring the sedimentation of macromolecules in a centrifugal field allows their hydrodynamic and thermodynamic characterization in solution, without interaction with any matrix or surface. An analytical ultracentrifuge can be used to perform two different types of experiments, termed sedimentation velocity and sedimentation equilibrium.

Sedimentation velocity experiments measure the velocity and diffusion of a lamella of macromolecules that is formed in solution under the influence of a strong centrifugal field (250000*g*). These fields are sufficient to force particles as small as several nanometers in diameter to sediment during several hours or less. During the sedimentation process, the concentration profiles in the sample cell are registered by optical systems (absorbance, RI), providing information on mass, density, and shape, the three basic properties that govern a particle's sedimentation velocity.

Additionally, concentration and diffusion effects oppose sedimentation, where the latter is time-dependent. Sedimentation equilibrium experiments are performed at lower speeds and measure the equilibrium concentration distribution of macromolecules that are formed when the transport by sedimentation is balanced by diffusive transport. The determination of the sedimentation equilibrium of macromolecules is the method of choice for molar mass determinations and the study of self-association and heterogeneous interactions, such as protein−protein, protein−nucleic acid, and protein−small molecule binding.

The combination of new instrumentation and powerful computational software for data analysis results in easy AU experiments with reproducible results. AU also provides the ability to perform analysis with relatively high throughput, although the sample must be as pure as possible. AU can be applied to aqueous and nonaqueous systems, with detection at any wavelength across the UV−visible spectrum (*181*). Moreover, AU allows recovery of the analyzed sample. On the other hand, AU instrumentation is expensive to purchase.

AU is often applied during manufacture, formulation, and quality control of biopharmaceuticals to provide information concerning aggregation and biologically important molecular interactions; therefore, AU could also be a suitable technique to analyze NDS as these systems can be based on assembled proteins. In fact, the particle size distribution and the shape of human serum albumin NDS in aqueous solutions have already been studied by sedimentation velocity analysis in the analytical ultracentrifuge (*46*). AU is also a promising technique for characterizing nanoliposomes, as AU has been used earlier for assessing submicrometer liposome size and size distribution (*182*). This holds also for polysaccharide-based NDSs in view of former studies on polysaccharides and liposaccharides−chitosan complexes via AU analysis (*183, 184*).

5.4. NMR Spectroscopy. NMR is based upon the measurement of absorption of radiofrequency radiation by atomic nuclei with nonzero spins in a strong magnetic field (*185*). The absorption of the atomic nuclei is affected by the surrounding atoms, which cause small local modifications to the external magnetic field. In this way, detailed information about the molecular structure of a food sample can be obtained. Among nuclei with nonzero spin, the isotopes of hydrogen-1 (spin = 1/2) and carbon-13 (spin = 1/2) are the most used in NMR, although other isotopes such as nitrogen-15 (spin = 1/2), oxygen-17 (spin = 5/2), fluorine-19 (spin = 1/2), or phosphorus-31 (spin = 1/2) are also frequently employed.

The main characteristics of NMR are (*186*) as follows: It is a nondestructive method that makes it possible to perform different analyses on the same sample; it is able to detect different nuclei, allowing a study of the sample under different perspectives; it is structure-sensitive, that is, capable of investigating structural features in the solution phase and solid phase; and it is sensitive to dynamics, which allows differentiation between molecules or portions of molecules with different mobility. NMR techniques are particularly useful for studying the structure and conformation of macromolecules in food. In food analysis, two types of NMR are applied, low-resolution NMR (LR-NMR) and high-resolution NMR (HR-NMR) (*185*). Nowadays, LR-NMR instruments (using frequencies of 10−40 MHz) are small, easy to use, and relatively inexpensive, which make them suitable to perform rapid and reproducible measurements. However, LR-NMR requires reference methods to carry out quantitative analysis, and in many cases, the precision of such reference method is a limiting factor. The advantage of HR-NMR (using frequencies above 100 MHz) over LR-NMR is that it is possible to obtain much more detailed information regarding the molecular structure of a food sample (*187*). The major disadvantage is that it is one of the most expensive analytical techniques to employ, in terms of both initial capital outlay and running costs. Next to this, extraction procedures may be necessary to enrich the studied compound as the sensitivity of HR-NMR is rather poor (*185*). Moreover, the structural characterization by NMR becomes more difficult when the size of the molecule increases and the NMR analysis is rather slow.

With respect to polysaccharide-based NDS, NMR has been applied earlier to characterize chitosan and its derivatives and cyclodextrins (*178, 188, 189*). NMR has also been used to investigate the physicochemical properties of lipid NPs (*79*). As NMR is a suitable technique to study protein aggregates (*186, 190*), it is expected that protein-based NDS can be investigated as well. However, the limiting factor here concerns the size of the aggregates.

5.5. X-ray Diffraction (XRD). XRD is used to determine the identity of crystalline solids based on their atomic structure. Diffraction occurs because X-rays have wavelengths in the order of a few angstroms, which are about the same as the interatomic distances in crystalline solids. A diffractometer can be used to make a diffraction pattern of a crystalline solid. During this XRD analysis, X-ray beams are reflected off the parallel atomic layers within a molecule over a range of diffraction angles. Because the X-ray beam has a single specific wavelength, constructive or destructive interference can occur. At certain angles, the reflected rays are in phase (constructive interference), and this will give a peak in a diffractogram. From the diffraction pattern, one can identify the molecule or mixture of molecules. More or less like a "fingerprint", every molecule has its own distinct set of diffraction peaks that can be used to identify it. Identification of the molecules is usually done by comparing the measured diffractogram with a database of know diffraction data.

XRD is a versatile, nondestructive technique that reveals detailed information about the chemical composition and crystallographic structure of natural and manufactured materials. X-ray crystallography yields unparalleled high-resolution structures of biomolecules and complexes from the solid phase (*191*). In fact, with this technique, it is possible to determine the position of the atoms more accurately (factor 100−1000) than with AFM. In general, the structures obtained with XRD agree with structures obtained with NMR, although there have been exceptions. XRD requires relatively large quantities of material and is slow. These materials may be precious, that is, difficult

Table 1. Overview of Several Characteristics of the Analytical Techniques That Can Be Used for Separating, Imaging, and/or Characterizing NDS in Food Products[a]

techniques	sensitivity	simplicity	time analysis	costs	reported applications	types	parameters
			separation				
HPLC	±	+	±	+	+	l, p, s	size
FFF	±	+	+	+	±	l, p, s	size
HDC	±	+	+	+	−	l, p, s	size
CE	−	+	±	+	±	l, p, s	size, charge
gel electrophoresis	±	+	−	+	±	p	size
			imaging				
TEM	+	+	−	−	+	l, p, s	(surface) structure
SEM	+	+	−	−	±	l, p, s	(surface) structure
AFM	±	+	±	−	±	l, p, s	(surface) structure
			characterization				
MALDI-MS	+	±	−	−	+	l, p, s	mass, composition
ESI-MS	+	+	+	±	±	p, s	mass, composition
DESI-MS	+	±	+	−	−	−	mass, composition
IM-MS	+	±	+	−	±	l, p, s	mass, composition
PCS	±	+	±	+	+	l, p, s	size (distribution)
AU	±	±	−	−	±	l, p, s	size, shape, structure
NMR	−	±	±	−	±	l, p, s	composition
XRD	+	−	−	−	±	l, p, s	structure
SAXS	±	−	−	−	−	−	size, shape, structure

[a] The characteristics include the sensitivity and simplicity of the analytical tool, time of the analysis, instrumental costs, number of reported applications, types of NDS [lipid-based (l), protein-based (p), or polysaccharide-based (s)], and parameters measured. Favourable (+), moderate (±), and unfavourable (−). For abbreviations of the techniques, see sections 3−5.

to obtain or produce. On the other hand, the size of the crystals needed is still decreasing because of new XRD developments.

It is demonstrated in various studies that XRD is a suitable technique to investigate the structure of NDS. For example, XRD has been used to characterize different types of lipid nanocapsules (*79, 192−194*). Concerning protein-based nanocapsules, XRD has already been applied to study albumin−zinc NPs, albumin−protamine−oligonucleotide NPs, and lactoglobulin aggregates (*195−197*). Finally, polysaccharide (coated) NPs have also been characterized before with XRD (*178, 198, 199*).

5.6. Small-Angle X-ray Scattering (SAXS). Traditionally, particle size analysis has been performed using light scattering, where the interference patterns from lasers are used to measure particle size. As the wavelength of visible light is 0.4−0.7 μm, these methods have a lower limit of 100 nm and are therefore not appropriate for studying NPs. However, X-rays, with a wavelength range of 0.01−1 nm, are ideal for this purpose; therefore, SAXS is suitable for structure analysis of materials (*200*). SAXS enables the determination of the structure of a variety of objects such as solutions of biological macromolecules, nanocomposites, alloys, synthetic polymers, and possibly also NDS (*201*). Nagoa et al. determined scattering profiles of gold NPs prepared by different methods using SAXS (*202*).

In a SAXS instrument, a monochromatic beam of X-rays with wavelengths of 0.1−0.2 nm is brought to a sample from which some of the X-rays scatter, while most simply go through the sample without interacting with it. The scattered X-rays, resulting from inhomogeneities in the nanometer range and recorded at very low angles, typically 0.1−10°, form a scattering pattern that is then detected at a detector, which is typically a two-dimensional flat X-ray detector situated behind the sample perpendicular to the direction of the primary beam that initially hit the sample. The scattering pattern contains information on the structure, shape, and size of macromolecules and the surface-to-volume ratio of particles. The method is accurate, nondestructive, and usually requires only a minimum of sample preparation.

Basically, the problem in SAXS data analysis is to get a three-dimensional structure from an one-dimensional scattering pattern. In the past, only overall particle parameters (e.g., volume, radius of gyration) of the macromolecules were directly determined from the experimental data, whereas the analysis in terms of three-dimensional models was limited to simple geometrical bodies. The 1990s brought a breakthrough in SAXS data analysis methods, allowing reliable ab initio shape and domain structure determination and detailed modeling of macromolecular complexes using rigid body refinement (*203, 204*). Nowadays, laboratory instruments have become available using advanced software capable of analyzing nanostructures in the range of 1−100 nm including shape and orientation distributions in inorganic nanopowders. SAXS was used to characterize the size and aggregation behavior of the Pt NPs (*205*) and crystal size and shape of Pt NPs (*206*). Characterization of NPs or NDSs using SAXS in other matrices than the bulk material itself has also been reported but appears to be limited (*207*).

6. CONCLUSIONS

Currently, a lot of attention is being paid to the developments in the area of nanotechnology. Potential NDSs for food applications are biodegradable polymers based on lipids, proteins, or polysaccharides and are described in this review. From a food safety point of view, nanofoods will face some major restrictions. Although NDSs may offer interesting technological solutions for food processing, contribute to improved sensory properties of food, and may exert health benefits, little is known about the in vivo effects. To evaluate the in vivo properties of these NDSs, NDSs need to be analytically characterized under consumption or other in vivo conditions. This review presents a comprehensive description of those analytical techniques that are currently used, or may be used, for the identification and/or characterization of NDSs in food applications. These techniques are divided into three classes: imaging, separation, and characterization techniques. An over-

view of these analytical techniques is presented in **Table 1**. It is clear that all techniques have advantages and limitations.

The imaging techniques discussed in this review are TEM, SEM, and AFM. Although these imaging techniques give a good representation of the (surface) structure of the NDS, they are generally very expensive and not suitable for routine analyses. Furthermore, sample preparation before imaging may affect the NDS structure. The separation techniques that are considered are several types of chromatography and electrophoresis. It should be noted that the reviewed techniques are intended for separating NDS from the food matrix. On the basis of sensitivity, analysis time, costs, reported applications, types of NDSs, and parameters that can be measured, it is suggested that for the size- or charge-based separation of NDSs, HPLC and FFF are the methods of choice.

An accurate characterization of NDSs, for example, size, charge, shape, structure, and composition, following separation from the food matrix is feasible; however, a combination of different techniques will be required. The characterization techniques reviewed for NDSs are MS, PCS, AU, NMR, XRD, and SAXS. As in many fields of analytical chemistry, MS is also a technique with large potential for the analysis of NDSs. While MS gives information about the mass and chemical composition, PCS is a complementary nondestructive method for the determination of NDS particle diameter and size distribution.

In conclusion, chromatographic techniques like HPLC or FFF combined with online PCS and MS appear to be a convenient option for the determination of the physical and chemical compositions of a broad range of NDSs in food applications. For specific questions, other techniques may be used.

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