Chapter 5
Protein-Based Nanoparticles

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5.1 Introduction

Proteins are linear polymers of L-α-amino acids with a wide variety of structures and functions. They can be classified in terms of their solubility, chemical structure, shape, and number of monomeric units.

Protein solubility is determined by a variety of interactions including protein–protein, protein–water, protein–ion, and ion–water interactions (Trevino et al. 2008). These biomacromolecules can be classified, based on their solubility in water-soluble proteins (albumins), salt-soluble proteins (globulins), alcohol-soluble proteins (prolamins), acid- or base-soluble proteins (glutelins), and insoluble in most solvents proteins (scleroproteins). While originally used for wheat and cereals, this adaptation of the Osborne fractionation scheme has been applied to most food proteins (Bean and Lookhart 2000). Taking in consideration the different physicochemical properties, biocompatibility, and degradability of food proteins, they have a potential role in the development of nanoparticles as nutraceutical delivery systems (Sundar et al. 2010). There is a growing interest in the use of nanoparticles for drugs and bioactive compounds delivery vehicles (Mohanraj and Chen 2006; Kreuter 2007). Protein nanoparticles are relatively

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easy to prepare and due to their subcellular size they can penetrate into tissues through the capillaries or be taken up by the cells (Chen et al. 2006). Desai et al. (1996) have shown that the efficiency of intestinal uptake of 100 nm nanoparticles is 15–250 times better compared to larger size particles. Encapsulation of hydrophobic bioactive compounds in protein nanoparticles usually increases their water solubility and bioavailability (Huang et al. 2010). Additional protection of some bioactive compounds is achieved through the intrinsic antioxidant activity of proteins (Elias et al. 2008).

Casein micelles are one of the naturally occurring protein-based nanosystems which allow a better and more efficient transport of dairy calcium, phosphate, and protein (Müller-Buschbaum et al. 2007). The micelles are formed by the four main types of caseins (α_{s1}, α_{s2}, β, and κ) and have an average diameter of 150 nm (Kaya-Celiker and Mallikarjunan 2012). However, currently there are several protocols for the preparation of nanosystems based on food proteins such as albumins, dairy proteins, gelatin, and some plant proteins. There are two main methods for the preparation of protein nanoparticles: emulsification and desolvation. In the first method, an aqueous solution of the protein is emulsified with a vegetable oil at room temperature and added to preheated oil dropwise to evaporate the water and form the nanoparticles (Jahanshani and Babaei 2008). In the second method, the dropwise addition under stirring of desolvating agents such as organic solvents or salts separates and coacervates the proteins in the aqueous phase. Finally, the addition of a glutaraldehyde solution will induce intraparticle cross-linking to form the protein nanoparticles (Jun et al. 2011). Self-assembly methods have also been reported.

5.2 Casein

Esmali et al. (2011) described the self-assembling of β-casein in micelles to be used as a nanovehicle for curcumin. This hydrophobic phenolic compound is very important in foods as antioxidant but it has also found important applications as antimicrobial since it has bactericidal activity against bacteria such as *Helicobacter pylori* (De et al. 2009). This self-assembly tendency of bovine caseins has also been used by Semo et al. (2007) to load liposoluble vitamin D2 into reassembled casein micelles. These nanoparticles can easily be included in dairy products without modifying their sensory properties and providing partial protection to the vitamin against UV light. A similar technique was used by Zimet et al. (2011) to load casein micelles with the omega-3 polyunsaturated fatty acid docosahexaenoic acid (DHA). Reassembled micelles prepared at 4 °C in the presence of calcium (50–60 nm) or at room temperature in the absence of it (288.9 nm) were able to protect DHA against oxidation showing good stability at 4 °C. Mohan et al. (2013) showed the ability of unmodified casein micelles to bind hydrophobic molecules such as vitamin A without having to perform the process of reassembling of the caseins. The elaboration of casein nanoformulations by enzymatic cross-linking by transglutaminase for entrapping bioactive molecules have also been reported (Elzoghby et al. 2011).

5.3 β-Lactoglobulin (β-LG)

β-LG is the main bovine whey protein, is a relatively small protein formed by 162 amino acids with a molecular weight of 18.4 kDa (Kontopidis et al. 2004). Nanoparticles with an average diameter of 60 nm have been prepared using a desolvation method which included a preheating at 60 °C of the β-LG solution, the use of acetone as desolvating agent, and glutaraldehyde as the cross-linking agent. The nanoparticles were stable under acidic and neutral conditions showing good potential for encapsulation and controlled delivery purposes (Ko and Gunasekaran 2006; Gunasekaran et al. 2007). The preparation of microparticles composed of a core of aggregated β-LG covered by a shell of carboxymethylcellulose (CMC) has been reported by Carpineti et al. (2014). The first step involves the formation of a core of aggregated β-LG by heating protein solutions at 80 °C for 15 min at pH 7 and then, in a second step, to induce the shell deposition by promoting the electrostatic union of the anionic CMC molecules on the previously formed core. The protein core has an average diameter of 200 nm which increases to 1 μm when the polysaccharide is incorporated.

5.4 α-Lactalbumin (α-LA)

It is quantitatively the second most important protein in whey, has a molecular weight of 14.2 kDa, is able to bind calcium, and is the regulatory subunit of the enzyme lactose synthase (Kamau et al. 2010; Kaya-Celiker and Mallikarjunan 2012). Partial hydrolysis of α-LA with a bacterial protease from *Bacillus licheniformis* was shown to induce the formation of nanotubes by self-assembly of the generated peptides. These are the only food protein nanotubes and are formed in the presence of calcium at neutral pH (Kaya-Celiker and Mallikarjunan 2012). Graveland-Bikker et al. (2009) propose the existence of dimeric building blocks which are able to self-assemble into a 10-start, right-handed helix via β-sheet stacking to form the nanotubes. The kind of self-assembly depends strongly on the protein concentration. At α-LA concentrations >3 % and in the presence of calcium, long tubular structures with diameters of about 20 nm can be obtained. At lower α-LA concentrations, linear fibrils with diameters of 5 nm or random aggregates are produced (Kamau et al. 2010). α-LA nanotubes are very stable and due to their long linear structure can be used as viscosifying agents (Kaya-Celiker and Mallikarjunan 2012). The presence of an 8 nm cavity in these tubes could be useful for the possible encapsulation of nutraceuticals, vitamins, and prebiotics (Kamau et al. 2010). This method is applicable to other proteins as well and has been investigated in the immobilization of enzymes or to prepare analogues to muscle fiber (Weiss et al. 2006). Esmaelizadeh et al. (2011) also used enzymatic hydrolysis to produce very small α-LA nanoparticles. They used the endoproteinase Glu-C (V8 protease) from *Staphylococcus aureus* strain V8 to produce partial hydrolysis of ALA in the

presence of calcium or manganese. The resulting mixture was annealed for 2–4 h in a water bath at 50 °C. Under these conditions, nanospheres with diameters between 3 and 5 nm were formed.

Mehravar et al. (2009) used the desolvation process for the preparation of α-LA nanoparticles. They examined the effect of pH, temperature, and solvent on the size of the nanoparticles. They found that the use of acetone as the desolvating agent produced smaller particles than ethanol and that the use of higher values of pH and lower temperatures generated nanoparticles with larger diameters. In this study, nanoparticles with sizes between 102 and 454 nm were obtained. Mehravar et al. (2011) applied the Taguchi method to optimize the diameter of the nanoparticles. The optimal conditions for the production of particles were pH 2.5, 50 °C, and a stirring speed of 750 rpm for a nanoparticle diameter of less than 220 nm. Arroyo-Maya et al. (2012) studied the effect of several treatments on the capacity of ALA to form nanoparticles by the desolvation method with glutaraldehyde cross-linking. The use of acetone as desolvating agent allowed the authors to obtain nanoparticles with smaller sizes (152.3 nm) as shown in Fig. 5.1. The use of the solvent with a small polarity index (isopropanol) produced the particles with the largest hydrodynamic diameter (293.4–324.9 nm). Ethanol produced intermediate size particles (205.1–246 nm). These results could indicate that by carefully controlling the hydrophobic interactions it is possible to control the size of α-LA nanoparticles. The nanoparticles obtained with ethanol had an isoelectric point of 3.61 and are very stable at pH values > 4.8 (according to their zeta potential values) so they could be useful as bioactive compounds' nanocarriers in different foods. The nanoparticles were degraded by trypsin at pH 2 and pancreatin at pH 8 even after the cross-linking reaction.

5.5 Bovine Serum Albumin (BSA)

BSA is a protein with great potential for the preparation of nanovehicles with possible food applications since it is nontoxic and biodegradable. Many reports have dealt with the production of BSA nanoparticles, generally by the desolvation method using acetone with glutaraldehyde cross-linking (Sailaja and Amareshwar 2012). The preparation of size-controlled BSA nanoparticles is still a challenge for food nanotechnologists and there are different proposals. Sailaja and Amareshwar (2012) indicate that size control of BSA nanoparticles can be achieved by the intermittent addition of acetone. Jun et al. (2011) reported that the size and the surface area-to-volume ratio of BSA nanoparticles can be controlled by varying the protein concentration, pH, and NaCl content. They also indicate that the surface area-to-volume relationship is more useful as a parameter than diameter for comparative studies of nanomaterials. Calcium citrate-loaded BSA nanoparticles with diameters from 260 to 919 nm were prepared with this method.

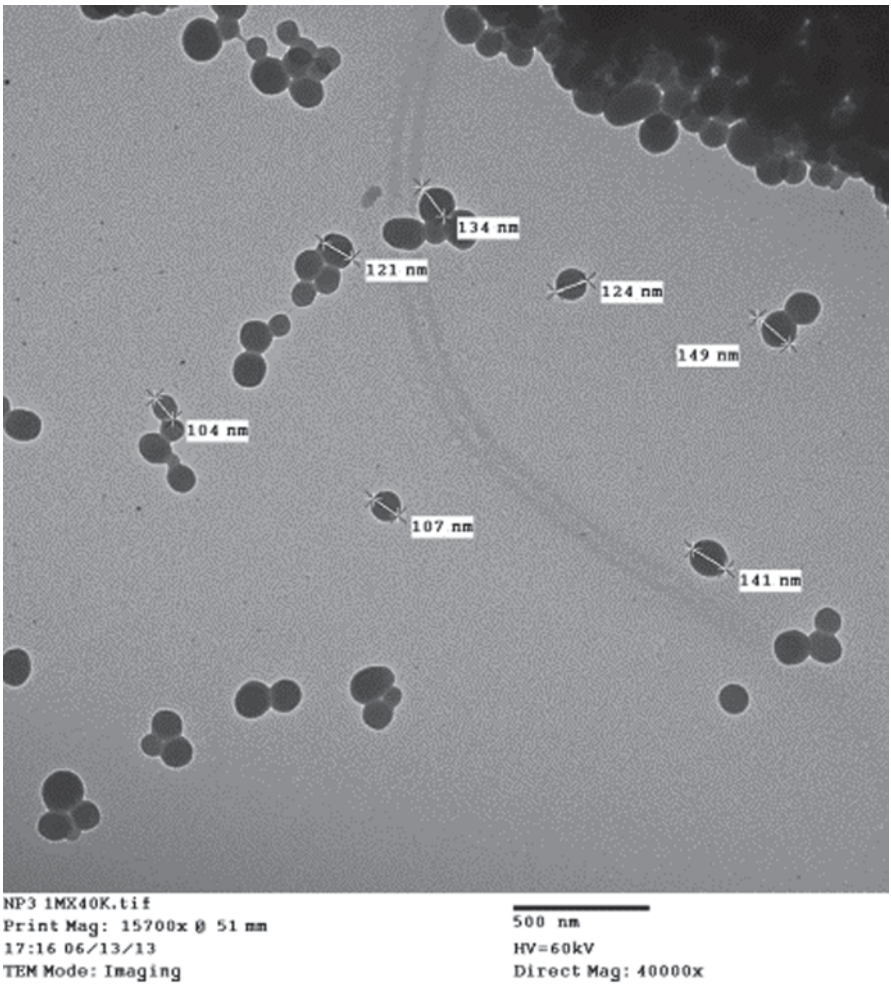

Fig. 5.1 α-Lactalbumin nanoparticles formed by desolvation using acetone as the desolvating agent at pH 3 and cross-linked with glutaraldehyde

Degradation by the enzymes of the gastrointestinal tract is a major factor influencing the efficiency of protein-based nanocarriers. Singh et al. (2010) used poly-L-lysine (PLL) to coat BSA nanoparticles, produced by desolvation with ethanol, to increase the resistance to in vitro enzymatic degradation. They found that the use of lower molecular weight PLL for coating increased the resistance to trypsin degradation and enhanced the stability of the BSA nanoparticles to be used as nutraceuticals nanovehicles.

5.6 Human Serum Albumin (HSA)

HSA, similarly to BSA, has been shown to be biodegradable, safe, easy to purify, and soluble in water, and therefore an ideal protein for nanoparticle preparation (Sebak et al. 2010). These nanoparticles have been usually prepared by the desolvation agent using ethanol as the desolvating agent. The nanoparticles have been stabilized by the addition of glutaraldehyde as a cross-linking agent or by heat denaturation (Weber et al. 2000). As in other cases, HSA nanoparticles have been characterized by sedimentation velocity analysis, dynamic light scattering, size exclusion chromatography, and electron microscopy (Vogel et al. 2002). Several studies have been performed on the parameters of the desolvation process. Results have indicated that the particle size depended on the amount of desolvating agent added (Weber et al. 2000) and on the pH value of the HSA solution prior to desolvation (Langer et al. 2003), but not on the amount of cross-linker of cross-linking procedure (glutaraldehyde or heat). At pH values of 8 and higher low-polydispersity, nanoparticles with average diameters between 200 and 300 nm can be prepared and the higher the pH, the smaller the nanoparticles. An increase in the concentration of glutaraldehyde reduces the surface amino groups decreasing the zeta potential of the nanoparticles (Langer et al. 2008). This kind of particle is degraded in the presence of different proteases such as pepsin and cathepsin B at low pH and trypsin and proteinase K at neutral pH. Arroyo-Maya et al. (2012) have indicated the importance of the dielectric constant and polarity index of the desolvating agent on the nanoparticle size for the case of α-LA. Storp et al. (2012) found something similar for the case of HSA and used a combination of methanol and ethanol as desolvating agent to prepare very small spherical HSA nanoparticles with diameters between 50 and 80 nm.

5.7 Egg Albumin (EA)

EA nanoparticles have also been prepared by the desolvation method using acetone as desolvating agent and cross-linking with glutaraldehyde (Taheri et al. 2012). Size and morphology were studied by photon correlation spectroscopy and atomic force microscopy. The process was optimized using the Taguchi method and the authors were able to obtain nanoparticles with a minimal diameter of 51 nm at 55 °C, 30 mg/ml EA concentration, 500 rpm agitation speed, and pH 4.

5.8 Gelatin

Gelatin is a natural protein prepared from collagen and is commonly used for pharmaceutical, food, and medical applications, because of its biodegradability and biocompatibility in physiological environments (Ramachandran and Shanmu-

ghavel 2010). Nanoparticles with diameters in the range of 112 to 386 nm have been prepared from this macromolecule by a two-step desolvation method using acetone or ethanol as desolvating agents and cross-linking with glutaraldehyde. These nanoparticles showed a good uptake by cells in culture (Azarmi et al. 2006). This method was originally described by Coester et al. (2000) indicating that after the first desolvation step, the low molecular gelatin fractions present in the supernatant are removed by decantation. The high molecular fractions present in the precipitate are resuspended and then desolvated again at pH 2.5 in a second step. The resulting nanoparticles can then be simply purified by centrifugation. Negatively charged gelatin nanoparticles have also been prepared by simple coacervation. These particles are spherical with a diameter of 45 ± 5 nm and are very stable (Mohanty et al. 2005).

5.9 Gliadin

Wheat gliadins are a highly polymorphic group of seed storage proteins which appear to be suitable polymers for the preparation of nanoparticles which are usually able to interact with biological surfaces such as the gastrointestinal mucosa (Jahanshani and Babaei 2008). Gliadin nanoparticles are usually prepared by the desolvation method using an ethanol:water phase (7:3 by vol.) as desolvating agent and cross-linking with glutaraldehyde. The particles so prepared have a diameter of 453 ± 24 nm, a zeta potential of 24.5 ± 0.5 mV, and a yield of 86.8 %. They developed a good bioadhesive interaction with the intestinal mucosa indicating a good potential as a nanovehicle for bioactive compounds (Arangoa et al. 2001). Ezpeleta et al. (1996) used a similar system to prepare all-*trans*-retinoic acid-loaded gliadin nanoparticles. They obtained 500 nm particles with a yield of about 90 % and an entrapment efficiency of about 75 % of the all-*trans*-retinoic acid. This compound has been widely used in the treatment of acne and other skin disorders.

5.10 Legumin

Legumin is one of the most important storage proteins in the pea seeds and is the source of sulfur-containing amino acids in these legumes (Jahanshani and Babaei 2008). Irache et al. (1995) prepared legumin nanoparticles of an average diameter of 250 nm by means of a pH-coacervation method and cross-linking with glutaraldehyde. Coacervates were prepared by mixing one volume of a 0.5 % w/v aqueous solution of legumin (pH 9 with NaOH 0.01 N) with two volumes of a constantly stirred solution of Synperonic™ PE/F 68 in phosphate buffer. The ionic strength and the pH were held constant at 0.103 M and 6.8, respectively. The nanoparticles were stable when the pH was neutral but were quickly degraded at low values of pH. Mirshahi et al. (2002) reported that these nanoparticles are not

able to elicit an immune response when injected intradermally in rats probably due to a reduction in antigenic epitopes of the legumin induced by the glutaraldehyde during cross-linking.

5.11 Zein

Zein, the corn prolamin protein, includes a group of alcohol-soluble proteins which are water insoluble (Gómez-Estaca et al. 2012). Zein nanoparticles are suitable, like gliadin nanoparticles, to incorporate hydrophobic bioactive compounds. There are reports of different methods to prepare this kind of nanoparticles. Zhong and Jin (2009) produced zein nanoparticles by liquid–liquid dispersion. This process takes advantage of the solubility characteristics of zein in ethanol at different concentrations. The particles were prepared by shearing zein solutions into an aqueous phase. The average size of zein nanoparticles was between 100 and 200 nm. This process has been used to encapsulate oregano, thyme, and cassia essential oils. Podaralla and Perumal (2010) produced zein nanoparticles by pH-controlled nanoprecipitation. The particles had an average particle diameter of 460 nm and were successfully used to encapsulate 6,7-dihydroxycoumarin. Gómez-Estaca et al. (2012) used electrodynamic atomization to form the zein nanoparticles. They studied the effects of protein concentration, flow rate, and applied voltage on the size and morphology of the particles. The diameter of the particles ranged from 175 to 900 nm and increased with zein concentration and flow rate. The adequate voltage for the process was 16 kV. Zein nanoparticles with round shapes were produced for zein concentrations from 5 to 15 %, however a sudden change to nanofibers was observed when the concentration reached 20 %. The particles were suitable to encapsulate curcumin. Xu et al. (2011) were able to produce hollow nanoparticles with mean diameters as small as 65 nm and capable of holding larger amounts of bioactive compounds to be carried into cells. In this method, a zein solution in 70 % ethanol was mixed with Na_2CO_3 precipitated in 70 % ethanol to wrap the zein. Water was added to precipitate the zein and to dissolve the carbonate leading to the formation of the hollow zein nanoparticles. Hydrophobic compounds encapsulated in this kind of particles showed a more constant and controlled release than the one in solid zein nanoparticles.

5.12 Soy Protein

Soy protein isolates (SPI) are adequate raw materials for the elaboration of nanoparticles. They consist mainly of the components glycinin and β-conglycinin. Curcumin-loaded SPI nanoparticles have been prepared by the desolvation method using ethanol as the desolvating agent and cross-linking with glutaraldehyde. The mean diameter of the particles was in the range of 220.1 and 286.7 nm and a zeta potential

of −36 mV which guaranteed a good stability. Encapsulation and loading efficiency were 97.2 and 2.7%, respectively (Teng et al. 2012).

5.13 Conclusion

Protein-based nanoparticles have shown a good potential as bioactive compound delivery systems for foods. So far, nanoparticles from different proteins including water-soluble (albumins, gelatin, SPI) and water-insoluble (zein, gliadin) have been successfully prepared and loaded with several bioactive compounds in order to increase their bioavailability and release. The balance in the composition of hydrophobic and polar or charged amino acids of the protein will have a strong influence in the type of bioactive compound which can be loaded into the nanoparticles. Albumins, for instance, have generally a low content of hydrophobic amino acids and their nanoparticles will be able to hold compounds by hydrogen bonds or electrostatic interactions. The opposite is true for gliadin or zein, and their nanoparticles are suitable for incorporating highly hydrophobic compounds. SPI have a balanced composition and therefore can form nanoparticles which can be loaded with hydrophilic or hydrophobic compounds. It is very likely that in the future chemically or genetically modified proteins with different amino acid balance could be available to prepare nanoparticles which can be loaded with almost any bioactive compound for controlled release to any organ and stable enough to be incorporated in diverse foods. Finally, caution has been suggested in some cases. There are reports indicating that some food processes that involve shear forces may provoke the formation of amyloid fibrils in high protein foods (Raynes et al. 2014). Toxicological testing is suggested in these special cases.

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