1 # Nanoencapsulation of food ingredients using protein-based delivery systems

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7 **Abstract**

8 Many proteins possess functional attributes that make them suitable for the encapsulation of bioactive agents,
9 such as nutraceuticals and pharmaceuticals. In this article, a state-of-the-art review of protein-based
10 nanoencapsulation approaches is given. The physicochemical principles underlying the major techniques for the
11 fabrication of nanoparticles, nanogels, and nanofibers from animal, botanical, and recombinant proteins are
12 described. Protein modification approaches that can be used to extend their functionality in these nanocarrier
13 systems are also described, including chemical, physical, and enzymatic treatments. The encapsulation, retention,
14 protection, and release of bioactive agents in different protein-based nanocarriers are discussed. Finally, some of the
15 major challenges in the design and fabrication of protein-based delivery systems are highlighted.

16 **Keywords:** Bioactive compounds; Nanoencapsulation; Protein; Release mechanism; Plant-based

17

1. Introduction

In recent years, considerable attention has been focused on the design of foods and food ingredients with enhanced nutritional and functional properties (McClements, 2015; Yao, McClements, & Xiao, 2015). Direct incorporation of certain types of bioactive agent into food products is challenging because of their low solubility, poor stability, and low oral bioavailability (McClements, Li, & Xiao, 2015; Simoes, et al., 2017). Nanoencapsulation is widely considered as a useful technology to enhance the stability and functionality of bioactive compounds (Katouzian & Jafari, 2016; Livney, 2015). In previous reviews, the nanoencapsulation of food ingredients using lipid-based (Fathi, Mozafari, & Mohebbi, 2012; Raikos & Ranawana, 2017; Salvia-Trujillo, Soliva-Fortuny, Rojas-Grau, McClements, & Martin-Belloso, 2017) and carbohydrate-based (Dalmoro, Cascone, Lamberti, & Barba, 2017; Fathi, McClements, & Martín, 2014; Yang, Han, Zheng, Dong, & Liu, 2015) delivery systems were discussed. Each of these systems has advantages and disadvantages in terms of their functional attributes, food matrix compatibility, and suitability for large-scale production (McClements, 2014). Nanoencapsulation of bioactive agents in protein-based delivery systems has also attracted much attention because of their specific nutritional and functional attributes (Adjonu, Doran, Torley, & Agboola, 2014; Livney, 2010; Rajendran, Udenigwe, & Yada, 2016; Semenova, Anokhina, Antipova, Belyakova, & Polikarpov, 2014). Some of the most important functional attributes of proteins include: (i) *surface-activity* – the ability to adsorb to the surfaces of colloidal particles and stabilize them from aggregation (McClements & Gumus, 2016); (ii) *structure formation* – the ability to assemble into structures such as spheres, tubes, or fibers (Mezzenga & Fischer, 2013); (iii) antioxidant activity – the ability to inhibit oxidation through chelation or free radical scavenging (Samaranayaka & Li-Chan, 2011). Moreover, proteins have a diverse range of functional groups on their surfaces that enable them to interact with a range of different substances, thereby facilitating the fabrication of nanocarriers that can encapsulate both hydrophilic and hydrophobic food bioactives (Elzoghby, Abo El-Fotoh, & Elgindy, 2011; Pereira, et al., 2009).

The proteins generally used for nanoencapsulation include those derived from animal (such as casein, whey, gelatin, albumin, or silk proteins), botanical (such as zein, gliadin, soy, or pea proteins), or recombinant (such as ferritin and silk proteins) sources (Chen, et al., 2014c). In this review, the molecular, physicochemical, and functional properties of proteins from different sources are described, and then their ability to encapsulate bioactive agents for food applications is discussed. The potential of modifying protein properties using chemical, physical,

and enzymatic treatments so as to extend their functionality is also discussed. Moreover, the major fabrication methods and release mechanisms for protein-based nanoencapsulation technologies are highlighted.

2. Protein-based encapsulating materials

Natural proteins are biological macromolecules composed of strings of amino acids linked together by peptide bonds, which have evolved through evolutionary pressures to serve important biological functions, such as structure formation, signaling, transport, and enzyme catalysis (Whitford, 2005). Some of the molecular and functional features of proteins can be used to fabricate encapsulation and delivery systems (Table 1). In the remainder of this section, a brief discussion of the attributes of proteins from different animal, botanical, and recombinant sources is given.

2.1. Animal-based proteins

Animal-based proteins can be obtained from a variety of sources, including the muscles, organs, eggs, milk, and other secretions of animals, fish, and insects. In this section, the properties of encapsulation and delivery systems fabricated from some of the most commonly used animal proteins are discussed, including those from meat, milk, egg, and silk.

2.1.1. Meat Proteins

The tissues of animals contain a number of proteins that can be used to assemble protein-based encapsulation and delivery systems, with the most important being collagen and gelatin (Aspevik, et al., 2017; Liu, Nikoo, Boran, Zhou, & Regenstein, 2015). Collagen is a hydrophilic biopolymer composed of three helical polypeptide chains that are about 1.5 nm in diameter and 400 nm in length, which can associate into triple helical and fibrous structures {Yu, 2011 #1}. Nevertheless, to the authors knowledge, collagen itself has not been widely used to develop colloidal delivery systems, which may be due to the difficultly in trapping bioactive molecules within it (Chan, So, & Chan, 2008; Friess, 1998). On the other hand, gelatin, which is derived from collagen, has been frequently utilized to create encapsulation and delivery systems.

Gelatin is typically obtained from collagen by either acid (type-A) or alkaline (type-B) hydrolysis (Ward & Courts, 1977). Powdered gelatin swells in cold water and becomes soluble in hot water (Li, Wang, & Wu, 1998). When a hot gelatin solution is cooled to a temperature sufficiently below its gelling point (around 35 ºC for

71 mammalian gelatin and around 10 ºC for fish gelatin), a strong hydrogel is formed {Ward, 1977 #3}. The strength
72 of the hydrogel formed depends on the gelatin concentration and type, solution conditions (pH and ionic strength),
73 and the thermal history (temperature-time profile) of the system. Conversely, when a gelatin hydrogel is heated
74 above its melting point (usually a few degrees above the gelling point) it will turn back into a liquid sol. Gelatin
75 therefore forms thermo-reversible hydrogels.

76 Gelatin nanoparticles have been prepared using a variety of fabrication methods, including
77 desolvation/coacervation (Lu, Yeh, Tsai, Au, & Wientjes, 2004a; Singh & Chaudhary, 2010), emulsion templating
78 (Bajpai & Choubey, 2006), and salting out (Hussain & Maji, 2008). However, the first two methods are the most
79 commonly used. Desolvation/coacervation is a process whereby a homogeneous solution of gelatin undergoes a
80 liquid–liquid phase separation, leading to the formation of a polymer-rich dense phase at the bottom and a
81 transparent solution above. Salt or alcohol addition, encourages coacervation, leading to the formation of
82 nanoparticles (Kumari, Yadav, & Yadav, 2010; Lu, Yeh, Tsai, Au, & Wientjes, 2004b). In the emulsion templating
83 method, a preheated gelatin solution is mixed with the bioactive component to be encapsulated at a temperature
84 above the melting temperature of the gelatin (Li, et al., 1998) (Fig. 1). The resultant mixture is then added to an oil
85 phase and homogenized to form a water-in-oil emulsion consisting of gelatin-rich water droplets dispersed in an oil
86 phase. This emulsion is then cooled to gel the gelatin molecules, and then the resulting nanogel particles are
87 removed by centrifugation and washing with an organic solvent. Chen et al. (2010) produced self-assembled
88 catechin-gelatin nanoparticles by flush addition of a gelatin solution into a stirred catechin solution at room
89 temperature (Chen, et al., 2010).

90 A number of different process parameters have been shown to influence the properties of the nanoparticles
91 formed from gelatin, including gelatin molecular mass, solution pH, and cross-linking. Traditionally, the strength of
92 gelatin gels is quantified in terms of the empirical Bloom test: the higher the Bloom value, the stronger the gel.
93 Application of gelatin with a higher Bloom value has been shown to lead to the formation of nanoparticles with
94 smaller sizes and slower release properties at low pH (Saxena, Sachin, Bohidar, & Verma, 2005). The effect of
95 production pH on the size of gelatin nanoparticles was studied by Van der Voort and Ludwig (2004), who indicated
96 that cross-linking reactions would be favored at higher pH (6), leading to a denser network and a reduction in
97 particle size in comparison to at lower pH (4). The addition of a chemical cross-linker, such as glutaraldehyde, was

98 shown to lead to better gelatin stability, a denser structure, and a smaller particle size (Cascone, Lazzeri,
99 Carmignani, & Zhu, 2002).

100 The gelatin molecules in protein-based nanoparticles can be chemically modified before or after particle
101 formation to alter their functional attributes. For instance, the attachment of PEG chains to the surfaces of gelatin
102 particles has been shown to reduce their interaction with other particles and with enzymes, thereby leading to
103 increased resistance to proteolytic enzymes and enhanced uptake of bioactives in the blood stream and lymphatic
104 tissues (Kommareddy & Amiji, 2007; Tobío, et al., 2000). PEGylated gelatin was reported to be completely non-
105 toxic to cells even when used at relatively high concentrations (200 μg/mL) (Kaul & Amiji, 2002). Oleic acid-
106 conjugated gelatin nanoparticles were produced using a self-assembly method that were designed to increase the
107 encapsulation of lipophilic bioactives (Tran, Tran, Vo, Vo, & Lee, 2013).

108 Gelatin molecules have also been modified using other chemical substituents. For example, thiol groups have
109 been attached to gelatin to modulate its ability to control the release of bioactive molecules under highly reducing
110 environments (Kommareddy, et al., 2007). Under non-reducing conditions, disulfide bonds are formed between
111 gelatin molecules that hold the nanoparticles together, but under reducing conditions these disulfide bonds are
112 broken and the bioactive is released. Thiolated gelatin carriers might be useful for flavor and antibacterial release,
113 under the reducing environment of hard cheeses.

114 *2.1.2. Milk proteins*

115 Milk contains numerous different types of proteins with various molecular structures and functional attributes.
116 However, the two most prevalent proteins are the caseins (80%) and whey proteins (20%). The caseins are
117 characterized by a high degree of molecular flexibility, whereas the whey proteins are characterized by compact
118 globular structures.

119 *2.1.2.1. Casein*

120 Casein contains four main protein fractions: α_{S1}, α_{S2}, β and κ-casein, in proportions of about 4:1:4:1. The
121 individual casein molecules have molecular weights ranging from around 19 to 24 kDa and isoelectric points
122 ranging from around 4.1 to 4.5 {Scherer, 2016 #4}. In nature, casein molecules pack into colloidal particles, known
123 as casein micelles, that function as delivery systems for calcium and phosphate to the developing infant. Casein

124 micelles typically have diameters ranging from about 50 to 500 nm (150 nm average). The highly flexible structure
125 of protein molecules means that they tend to be thermostable (Elzoghby, et al., 2011), which is an advantage for
126 applications where good thermal stability is required (Elzoghby, Samy, & Elgindy, 2012; Sauer & Moraru, 2012).
127 Conversely, caseins tend to highly aggregate close to their isoelectric point and in the presence of multivalent
128 counter-ions, which can limit their application in some foods.

129 A number of fabrication approaches have been developed to create nanoparticles from caseins. Nanoparticles
130 can be produced by reassembling the casein micelle structure from isolated casein molecules. Initially, a non-
131 covalent binding of a hydrophobic bioactive and casein is achieved via dropwise addition of the bioactive dissolved
132 in an organic solvent (such as ethanol) into a sodium caseinate solution. Reassembly of casein micelles has been
133 performed by the addition of $C_6H_5K_3O_7$, K_2HPO_4 and $CaCl_2$ to a solution of casein-bound bioactives. Nanoparticles
134 are formed by pH adjustment (6.7-7.0) and volume increment by water (Semo, Kesselman, Danino, & Livney,
135 2007). This method with some modifications has been used for the encapsulation of β-carotene (Sáiz-Abajo,
136 González-Ferrero, Moreno-Ruiz, Romo-Hualde, & González-Navarro, 2013) and ω-3 polyunsaturated fatty acids
137 (Zimet, Rosenberg, & Livney, 2011). An encapsulation efficiency of close to 100% was reported for ω-3 loaded
138 casein nanoparticles (Zimet, et al., 2011). In this study, the sizes of the carrier particles were not affected by
139 pasteurization (74 ºC, 20 s).

140 Among the different casein fractions, β-casein showed the most promising properties for the development of
141 encapsulation and delivery systems (Bachar, et al., 2012; Shapira, Davidson, Avni, Assaraf, & Livney, 2012). The
142 strongly amphiphilic character of β-casein means that it can be used to encapsulate both hydrophilic and
143 hydrophobic bioactives (Bachar, et al., 2012; Berry & Creamer, 1975). β-casein undergoes self-association under
144 physiologic conditions, forming micelle-like structures in aqueous solutions. Individual β-casein molecules have a
145 radius of gyration (R_g) around 4.6 nm in solution, while casein micelles (which contain from 15 to 60 β-casein
146 molecules) have been reported to have R_g values ranging from about 7.3 to 13.5 nm {Barth, 2012 #5}. The critical
147 micelle concentration ranges from around 0.05% to 0.2% w/v, depending on temperature, pH, solvent composition,
148 and ionic strength {Maeda, 1997 #6}. β-casein micelles may therefore be used to encapsulated both pharmaceutical
149 and nutraceutical compounds (Bachar, et al., 2012; Shapira, Assaraf, Epstein, & Livney, 2010; Shapira, et al., 2012).

150 *2.1.2.2. Whey proteins*

151 Whey proteins are particularly suitable for creating encapsulation systems for bioactive molecules because of
152 their surface activity and ability to form hydrogels (Gunasekaran, Ko, & Xiao, 2007). In contrast to caseins, which
153 are thermostable, whey proteins undergo thermal denaturation when heated above temperatures around 70 ºC
154 (LaClair & The University of Wisconsin, 2008). Above this temperature, the proteins partially unfold and associate
155 with each other through hydrophobic attraction and disulfide bond formation. Whey protein-based systems have
156 been used for the encapsulation of β-carotene (López-Rubio & Lagaron, 2012a), caffeine (Gunasekaran, et al.,
157 2007), epigallocatechin-3-gallate (Shpigelman, Cohen, & Livney, 2012), bilberry extract (Betz, et al., 2012), date
158 palm pit extract (Bagheri, Madadlou, Yarmand, & Mousavi, 2013; Sadeghi, Madadlou, & Yarmand, 2013), and
159 probiotics (López-Rubio, Sanchez, Wilkanowicz, Sanz, & Lagaron, 2012b; Picot & Lacroix, 2004). Delivery
160 systems can be produced using whole whey proteins or their fractions e.g. β-lactoglobulin (β-Lg), α-lactalbumin (α-
161 LA), and bovine serum albumin (BSA).

162 *Whole Whey proteins:* Using water as solvent makes the whey proteins as excellent potential carriers for food
163 applications to be electrosprayed (López-Rubio, et al., 2012a) or electrospun (Sullivan, Tang, Kennedy, Talwar, &
164 Khan, 2014). Depending on the pH conditions used for nanoparticle formation, the molecular organization and final
165 properties of the carriers change, and therefore capsules with a wide functional flexibility can be tailored for
166 different food products. The pH-induced cold gelation is considered as a promising solvent free method to produce
167 nanoparticles for food applications (see section 0). In contrast to the heat-induced gelation, this method makes
168 possible to entrap the heat-sensitive nutraceuticals at ambient temperatures (Alting, de Jongh, Visschers, & Simons,
169 2002; Alting, et al., 2004). pH-induced cold gelation of whey proteins has been used for the nanoencapsulation of
170 palm pit extract (Picot, et al., 2004) and folic acid (Kim, Lee, Oh, Kim, & Park, 2010). The details of these methods
171 are provided below.

172 *β-lactoglobulin:* Native β-LG is a small globular protein with exceptional potential for delivery of acid
173 sensitive bioactives (especially hydrophobic and amphipathic compounds) due to its stability against acidic media
174 and proteolytic enzymes in the stomach (Takagi, Teshima, Okunuki, & Sawada, 2003). However, thermally-
175 denatured β-LG is digested within the stomach because the peptide bonds are more exposed. β-LG may exist as
176 monomers, dimers, and higher structures depending on solution pH, ionic strength, and temperature {Perez, 2014

177 #7}. Both native and thermally modified β-LG can be used for the development of nano-scale delivery systems. It
178 is hypothesized that this globular protein has 2 to 3 binding sites on its surface that an interact with hydrophobic or
179 amphipathic substances (Lynen, Van Thuyne, Borremans, Vanhoenacker, & Sandra, 2003; Zimet & Livney, 2009).
180 Different kinds of colloidal particles, including clumps, spheres and fibers, can be formed by heating β-LG above its
181 thermal denaturation temperature by controlling the solution ionic strength and pH, because these parameters control
182 the hydrophobic and electrostatic interactions between the protein molecules (de la Fuente, Singh, & Hemar, 2002;
183 Livney, Corredig, & Dalgleish, 2003). Thermally-modified β-LG has a more open structure with high antioxidant
184 properties due to the exposure of thiol groups. Heat-denatured β-LG has been reported to bind water-soluble
185 compounds, such as EGCG, with a higher affinity and better stability against oxidation than native proteins
186 (Shpigelman, Israeli, & Livney, 2011).

187 α-lactalbumin: Under appropriate conditions, partial hydrolysis of this globular protein has been shown to
188 promote its self-assembly into nanotubes (Balandrán-Quintana, Valdéz-Covarrubias, Mendoza-Wilson, & Sotelo-
189 Mundo, 2013; Ipsen & Otte, 2007). For instance, α-LA self-assembled into nanotubes with a diameter of about 20
190 nm and a cavity of about 8 nm at neutral pH, when it was pre-treated with an enzyme (*Bacillus licheniformis*) in the
191 presence of a cation (calcium) (Fig. 2). These nanotubes are formed due to a nucleation and growth mechanism that
192 causes peptide monomers to assemble into helical structures. The minimum concentration for nanotube formation
193 was reported to be 20 g/L. Below this critical concentration fibrillar and/or random aggregates were formed
194 (Graveland-Bikker & de Kruif, 2006; Ipsen & Otte, 2003; Otte, Ipsen, Bauer, Bjerrum, & Waninge, 2005). It has
195 been shown that α-LA nanotubes can withstand pasteurization temperatures (40 s at 72 ºC) and are effective as
196 thickening agents due to their high aspect ratio and stiffness (Graveland-Bikker, et al., 2006).

197 *Bovine serum albumin (BSA):* BSA is another globular protein found in whey that has the ability to form
198 encapsulation and delivery systems. Ko and Gunasekaran (2006) compared the ability of β-LG and BSA to form
199 nanoparticles by a desolvation method, using glutaraldehyde as a cross-linker. They reported that under similar
200 conditions, β-LG nanoparticles were smaller and more uniform than BSA nanoparticles, due to the weaker
201 hydrophobic interactions of β-LG in comparison to BSA. Due to the denser matrix structure and lesser amount of
202 basic amino acids in β-LG compared to BSA, β-LG nanocarriers were more stable in acidic and neutral
203 environments, with and without the addition of proteolytic enzymes (Elzoghby, et al., 2012). BSA-based

204 nanoparticles have been produced using various different methods, including coacervation (Langer, et al., 2003),
205 thermal gelation (Yu, Yao, Jiang, & Zhang, 2006), emulsion-templating (Yang, et al., 2007), electrospinning
206 (Wongsasulak, Patapeejumruswong, Weiss, Supaphol, & Yoovidhya, 2010), and nano-spray drying (Lee, Heng, Ng,
207 Chan, & Tan, 2011).

208 *2.1.3. Egg proteins*

209 Both egg white and egg yolk contain numerous different type of proteins that have functional properties that
210 make them suitable for fabricating encapsulation and delivery systems {Phillips, 2011 #10}. For instance, egg white
211 contains ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), and lysozyme (3.5%),
212 whereas egg yolk contains lipovitellins (36%), livetins (38%), phosvitin (8%), and low-density lipoproteins (17%).
213 However, egg ovalbumin is the most commonly used egg protein for encapsulation purposes. The native form of
214 this protein is relatively resistant to pepsin digestion in the stomach, which is an advantage for some applications.
215 Biopolymer nanoparticles produced from ovalbumin have recently been used to encapsulate and protect retinol
216 (Visentini, Sponton, Perez, & Santiago, 2017). Studies have also shown that egg proteins can be chemically
217 modified to enhance their functional attributes. Li and Gu (2014) conjugated ovalbumin with dextran using the
218 Maillard reaction to produce self-assembled nanoparticles. Curcumin-loaded protein nanogels were formed by
219 thermal denaturation of ovalbumin-dextran conjugates close to their isoelectric point (Feng, Wu, Wang, & Liu,
220 2016).

221 It has been shown that water-soluble bioactives can be loaded into albumin nanoparticles using various
222 methods: (i) incubation of bioactives formed and hardened albumin nanoparticles (shorter time and lower load); (ii)
223 incorporation of bioactives into the albumin solution prior to particle formation (shorter time and higher load); (iii)
224 incorporation of bioactives into a cross-linker solution (such as glutaraldehyde) prior to albumin nanoparticle
225 formation (longer time & higher load) (Merodio, Arnedo, Renedo, & Irache, 2001). The first method leads to
226 surface deposition of the bioactives, while the second and third methods lead to a fairly homogenous incorporation
227 of the bioactives inside the protein nanoparticles (Merodio, et al., 2001).

228 *2.1.4. Silk proteins*

229 Silk fibroins from the cocoons of the silkworm *Bombyx mori* or spider webs have recently been explored as a
230 versatile protein biomaterial for a wide number of applications (Lammel, Hu, Park, Kaplan, & Scheibel, 2010). The

231 two primary proteins that compose silkworm silk, fibroin and sericin, consist of 18 different amino acids:
232 predominantly glycine, alanine and serine. In contrast, spider silk primarily contains glycine and alanine-enriched
233 fibroin proteins. The diversity of chemical groups in silk proteins enables site-specific functionalization via ion
234 addition of chemical species. For example, the covalent decoration of silk fibroin fibers with the Arg-Gly-Asp and
235 parathyroid hormone was reported to improve the cytocompatibility of silk (Sofia, McCarthy, Gronowicz, &
236 Kaplan, 2001). The PEGylation process generally reduces the nonspecific protein binding, increasing the
237 hydrophilicity of fibroin. Copolymerization can also be applied to enhance the physicochemical properties of silk
238 nanoparticles (Wantanasiri, Ratanavaraporn, Yamdech, & Aramwit, 2014; Zhou, et al., 2013). Silks show the self-
239 assembly, mechanical toughness (high tensile strength, flexibility and resistance to compressive forces), and feasible
240 processing method, therein presenting considerable utility for the formation of films, fibers and nanospheres.

241 Silk based biomaterials generally exhibit process dependent biocompatibility (process of extraction or purification).
242 Concerns about their biocompatibility arouses from virgin silks, which might lead to hypersensitivity delay to acute
243 or chronic inflammatory (Altman, et al., 2003; Kundu, et al., 2014). However, by removing the silk sericin
244 (degumming), a minimal inflammatory tissue reaction was observed (Altman, et al., 2002). The typical degumming
245 process involves the treatment in sodium carbonate and sodium dodecyl-sulfate at high temperature and then
246 dissolving in calcium nitrate tetrahydrate and methanol solution (Gupta, Aseh, Ríos, Aggarwal, & Mathur, 2009).

247 Silk nanocarriers might be produced using different methods such as salting out (Lammel, et al., 2010),
248 electrospinning (Sheng, et al., 2013), electrospray (Wantanasiri, et al., 2014), gelation (Wu, et al., 2012a), liquid
249 anti-solvent precipitation (Subia & Kundu, 2013). Curcumin loaded nanoparticles were produced by noncovalent
250 reaction of silk fibroin and chitosan. The introduction of chitosan in the nanoparticles formulation of silk fibroin
251 resulted in an increase in its hydrophilic character, and therefore in the enhancement of their water solubility. A
252 sustained release was observed for a 8-day period (Gupta, et al., 2009). Due to their great features; e.g.
253 thermostability, mechanical resistance (Numata & Kaplan, 2010), and sustained release (Kundu, Chung, Kim, Tae,
254 & Kundu, 2010), silk based nanoparticles could be used for the encapsulation of flavor to extend their release in
255 chewing gums, or protect bioactives at cooking temperatures.

256 **2.2. Plant-based proteins**

257 Plant-based proteins can be obtained from a variety of botanical sources or their waste products. In the
258 following sections, different properties and modification methods of encapsulation and delivery systems fabricated
259 from some of the most commonly used plant proteins are discussed, including zein, soy, and pea proteins.

260 *2.2.1. Zein proteins*

261 Zein is a proline-rich storage protein that is found in corn {Patel, 2014 #192}. It contains about three quarters
262 hydrophobic and one quarter hydrophilic amino acid residues, which means that it tends to be insoluble in water, but
263 soluble in some organic solvents (such as concentrated ethanol solutions). Due to its high hydrophobicity, zein is a
264 particularly good building block for developing encapsulation and delivery systems for water-insoluble compounds,
265 such as essential oils (Wu, Luo, & Wang, 2012b), omega-3 fatty acids (Torres-Giner, Martinez-Abad, Ocio, &
266 Lagaron, 2010), vitamin D_3 (Luo, Teng, & Wang, 2012), curcumin {Huang, 2016 #176;Zou, 2016 #178;Patel, 2010
267 #194}, resveratrol (Davidov-Pardo, Joye, Espinal-Ruiz, & McClements, 2015), quercetin {Patel, 2012 #193}and
268 lutein (Hu, Lin, Liu, Li, & Zhao, 2012). The zein molecule has a "brick-like" shape that can self-assemble into
269 colloidal structures capable of carrying hydrophobic molecules in their interiors (Elzoghby, et al., 2012). It can also
270 be used for the encapsulation and release of water-soluble compounds such as nisin for more than 200 h (Xiao,
271 Davidson, & Zhong, 2011). Zein particles exhibit a limited digestion in the stomach, slow release in the small
272 intestine, and a more rapid release in the large intestine, and so they are particularly suitable for the delivery of acid-
273 sensitive compounds or for the sustained-release of antibacterial compounds in acidic foods (Donsì, Voudouris,
274 Veen, & Velikov, 2017).

275 Electro-hydrodynamic methods have also been used to develop encapsulation and delivery systems based on
276 zein, such as electro-spinning (Fernandez, Torres-Giner, & Lagaron, 2009) and electro-spraying (Gomez-Estaca,
277 Balaguer, Gavara, & Hernandez-Munoz, 2012) approaches. Other methods, such as those assisted by supercritical
278 fluids (Hu, et al., 2012) and spray drying (Chen & Zhong, 2014a) have also been used.

279 Bare zein nanoparticles are highly unstable to aggregation in aqueous solutions because they have a relatively
280 high surface hydrophobicity {Patel, 2014 #192}. However, their stability can be improved by coating them with
281 emulsifiers after formation, such as sodium caseinate (Davidov-Pardo, et al., 2015; Patel, Bouwens, & Velikov,
282 2010), β-lactoglobulin (Chen, Zheng, McClements, & Xiao, 2014b) or Tween 20 (Hu & McClements, 2014), so as

to cover the hydrophobic patches and generate electrostatic and steric repulsion. Alternatively, they can be protected from aggregation by adsorbing ionic polysaccharides to their surfaces, such as pectin (Hu, et al., 2015a) or alginate (Hu & McClements, 2015b), to increase the electrostatic and steric repulsion between them.

2.2.2. Soy proteins

Proteins isolated from soybeans have also been shown to exhibit functional properties, such as surface activity and structure formation, which make them appropriate for developing encapsulation and delivery systems (Endres, 2001). However, they must be carefully extracted and purified to avoid excessive protein denaturation and aggregation, since this can cause a loss of functionality. Soy protein nanoparticles have been produced using liquid–liquid dispersion (Teng, Luo, & Wang, 2012), cold gelation (Zhang, Liang, Tian, Chen, & Subirade, 2012), and controlled thermal denaturation {Zhu, 2017 #195} methods. For instance, various types of protein particles could be formed from soy protein isolates using the liquid-liquid dispersion method, including microspheres, hydrogels, and polymer blends (Teng, et al., 2012). Soy protein nanoparticles have recently been used to encapsulate □-carotene, which was attributed to the binding of hydrophobic carotenoid molecules to non-polar patches on the protein nanoparticle surfaces {Deng, 2017 #196}. Soy protein nanoparticles have also recently been used to encapsulate resveratrol, which improved its water-dispersibility, stability, and bioavailability {Pujara, 2017 #197}.

2.2.3. Pea proteins

Pea proteins consist of a mixture of different types of globular proteins, with a major fraction of globulins (65–80%) and minor fractions of albumins and glutelins {Owusu-Ansah, 1987 #8}. Within the globulin fraction, there are three main types of protein: legumin, vicilin and convicilin. Like many other globular proteins, pea proteins are surface active and structure forming properties, which makes them suitable candidates for forming encapsulation and delivery systems. The surfaces of native pea proteins are predominantly hydrophilic, which means that they tend to be water-soluble. Pea proteins have been used as emulsifiers to form and stabilize oil-in-water nanoemulsions produced by high-pressure homogenization (Donsì, Senatore, Huang, & Ferrari, 2010). In this case, the non-polar bioactive molecules are usually dissolved in the oil phase prior to forming the nanoemulsion, which leads to the generation of bioactive-loaded protein-coated lipid nanoparticles.

Pea protein-polysaccharide electrostatic complexes have been formed at pH values where the proteins and polysaccharides have opposite charges (Gharsallaoui, et al., 2012). The formation of these complexes provides new

310 functions to the pea proteins, without any chemical or enzymatic modification, particularly solubility, foaming, and
311 surface properties (Liu et al., 2010). The formation of interfacial pea protein-polysaccharides complexes can be
312 used to improve the stability of emulsions to aggregation by increasing the repulsive interactions between the lipid
313 droplets (Nesterenko, Alric, Silvestre, & Durrieu, 2013). Indeed, lipid droplets coated by pea protein-pectin
314 complexes have been shown to have better stability to ageing, pH changes, and spray-drying than those coated by
315 proteins alone.

316 **2.3. Recombinant-based proteins**

317 Recombinant proteins are manufactured using biological processes, usually inside host cells, such as mammals,
318 bacteria or yeast. It has been estimated that about 45% of recombinant proteins are produced using mammalian cell
319 hosts, 39% using bacterium *Escherichia coli,* and 15% using the yeast *Saccharomyces cerevisiae* (Sanchez &
320 Demain, 2012). A major advantage of using recombinant proteins is that a specified protein can be produced in
321 higher purity than is normally possible naturally. However, there may be issues with consumer acceptability and
322 large-scale economical production.

323 As mentioned earlier, silk proteins can be used for the encapsulation, protection, and delivery of bioactive
324 agents. However, large-scale production of this protein using conventional farming methods is not feasible. In this
325 case, the production of silk proteins using recombinant methods may be more sustainable than using natural
326 alternatives. The host organisms could be bacteria, yeast, as well as insect cells, plant cells, mammalian cells, and
327 even transgenic mammals (Tokareva, Michalczechen-Lacerda, Rech, & Kaplan, 2013).

328 Ferritins are a class of iron storage proteins that are present in animals, plants and bacteria {Wang, 2010 #1}.
329 They have a cage-like structure that is ideal for the encapsulation of bioactives that have relatively small dimensions
330 (around 12 nm). For instance, curcumin has been encapsulated into recombinant human H-chain ferritin hosted in
331 *Escherichia coli* (Chen, et al., 2014c). It should be stressed that recombinant proteins are only likely to be suitable
332 for specialist high-value applications, rather than general use.

333 **3. Production methods and modification**

334 In the following section, a brief overview of different technologies that can be used to fabricate nanoscale
335 delivery systems for based on proteins is provided.

3.1. Protein modification for encapsulation

Prior to creating nanoparticles, proteins can be modified to enhance their functional performance. The modification method could be performed (i) chemically (carbohydrate or lipid conjugation and cationization), (ii) physically (sonication), or (iii) enzymatically (crosslinking). In this section, an overview of some commonly used methods of modifying protein properties is given.

3.1.1. Protein-carbohydrate Conjugation

The Maillard reaction typically occurs between a molecule with a carbonyl group (usually a reducing sugar) and another molecule with an amino group (usually an amine, amino acid, peptide, or protein) (Troise & Fogliano, 2013). The conjugation reaction is based on mixing a solution of proteins and carbohydrates under controlled pH conditions, and then drying the resulting solution at a controlled temperature and relative humidity for a fixed time. This type of chemical modification of proteins can be used to improve their emulsifying properties, water-solubility, antibacterial activity, and reduce their allergenicity {de Oliveira, 2016 #9}. Protein-carbohydrate conjugates are typically stable over a greater range of pH values than unmodified proteins {Kato, 2002 #10}. In addition, the antioxidative properties of proteins are often enhanced when they are conjugated with carbohydrates, which improves their ability to retard lipid oxidation. Maillard conjugates have been used in the encapsulation of essential oils (Shah, Davidson, & Zhong, 2012) and fish oils (Augustin, Sanguansri, & Bode, 2006). {Li, 2017 #2} studied use of sodium caseinate-lactose complex, conjugated via the Maillard reaction for oil encapsulation. The produced conjugated showed better emulsifying properties and stability. {Muhoza, 2017 #3} reported use of glycosylated casein by Maillard reaction with dextran for delivering coenzyme Q10. Conjugated casein showed better stability at pH around the pI. The glycosylated casein micelles size were dependent on the grafting degree.

3.1.2. Protein-lipid Conjugation

Lipid molecules can also be covalently attached to protein molecules to alter their functional attributes. For instance, proteins can be conjugated to fatty acid chains using an acylation reaction, which increases their hydrophobicity, surface-activity, and emulsifying capacity {Wu, 2014 #11}. Incorporation of hydrocarbon chains into the protein macromolecules leads to formation of amphiphilic compounds with improved surface activity {Rondel, 2009 #4}. Grafting dodecanoyl chains to soy proteins led to the formation of vitamin E-loaded protein

362 microcapsules with smaller dimensions and better encapsulation efficiency than soy proteins alone (Nesterenko,
363 Alric, Silvestre, & Durrieu, 2014a).

364 *3.1.3. Protein cationization*

365 The electrical characteristics of proteins can be altered using a variety of methods. For instance, cationization
366 can be used to enhance the solubility, swelling power, and water absorption properties of proteins {Futami, 2005
367 #6}. Proteins cationized by chemical conjugation also show efficient intracellular delivery via adsorptive-mediated
368 endocytosis and then can exert their biological activity in cells. Cationization of native proteins can be performed by
369 amidation of carboxylates with diamines, conjugation with a cationic polymer and coupling to polycationic peptides.
370 The denatured proteins are cationized by S-alkylation of cysteine residues and S-alkylsulfidation of cysteine
371 residues {Futami, 2007 #5}. This type of modification has been shown to increase the encapsulation efficiency of
372 hydrophilic compounds, while the encapsulation efficiency of hydrophobic compounds decreases (Nesterenko, et
373 al., 2014a; Nesterenko, Alric, Violleau, Silvestre, & Durrieu, 2014b).

374 *3.1.4. High-intensity Ultrasonic Modification*

375 High-intensity ultrasonic (sonication) methods can be used to alter the molecular characteristics of proteins,
376 and therefore alter their functional properties. Improvements in solubility, gelling ability, and specific surface area
377 of whey protein (Jambrak, Mason, Lelas, Herceg, & Herceg, 2008; Zisu, Bhaskaracharya, Kentish, & Ashokkumar,
378 2010) and soy protein (Jambrak, Lelas, Mason, Krešić, & Badanjak, 2009) have been reported as a result of
379 sonication. {Stathopulos, 2004 #4} reported that sonication of a range of structurally diverse proteins led to
380 formation of aggregates that have similarities to amyloid aggregates due to a wide range of protein conformational
381 disorders. This treatment caused formation of fibrils with diameters of 5–20 nm.

382 *3.1.5. Enzymatically modification*

383 Protein modification can be performed enzymatically that possesses some advantages such as fewer by-products,
384 milder process conditions, and easy handling. Degree of hydrolysis is the main factor affecting functional properties
385 of modified proteins. Extensive hydrolysis is helpful to increase the nutritional and therapeutic value of proteins
386 while limited hydrolysis that is more favorable for encapsulation poupuses, improves the functional properties
387 {Barać, 2011 #7}{Polanco-Lugo, 2014 #8}. {Zhao, 2009 #9} studied the effect of two enzymes (trypsin and
388 neutrase) on soy protein emulsifying activity at 1 and 2% degree of hydrolysis. Peptide composition change was

389 reported as a function of enzymes as well as degree of hydrolysis. Hydrolysates with 1% degree of hydrolysis
390 obtained by trypsin treatment showed better emulsifying activity due to the formation of larger peptides. There are
391 limited data on application of enzymatically modified proteins as shell materials, while they showed high potential
392 to be used by coacervation method.
393

394 **3.2. Encapsulation technologies**

395 A wide variety of fabrication technologies have been used for the nanoencapsulation of bioactives using
396 protein-based materials. Table 2 summarizes the main advantages and disadvantages of some of the most
397 commonly used fabrication technologies for this purpose.

398 *3.2.1. Coacervation*

399 Coacervation is a phase separation process that can occur within a polymer solution when there is a sufficiently
400 high attractive force between the polymers. It usually leads to the formation of two separate liquid phases: a
401 polymer-rich phase (coacervate) and a polymer-depleted phase (equilibrium solution) {Timilsena, 2017 #12}. In
402 aqueous solutions containing a mixture of two biopolymers (such as a protein and a polysaccharide), coacervation
403 usually occurs because of an electrostatic attraction between oppositely charged groups on the different biopolymers
404 (Koupantsis, Pavlidou, & Paraskevopoulou, 2014; Yan, et al., 2014).

405 The encapsulation of hydrophobic bioactive components using complex coacervation usually involves a
406 number of steps (Fig. 3). First, the bioactive agent is dissolved in an oil phase that is then homogenized with an
407 aqueous phase containing an emulsifier, which leads to the formation of an oil-in-water emulsion containing
408 bioactive-loaded lipid droplets. Second, the emulsion is mixed with an aqueous solution containing two
409 biopolymers (usually a protein and an ionic polysaccharide) at a pH where they have similar charges. In the case of
410 a protein and an anionic polysaccharide, this would be a pH above the isoelectric point of the protein. Conversely,
411 for a protein and a cationic polysaccharide, this would be a pH below the isoelectric point of the protein. Third, the
412 pH of the system is adjusted so that there is an attractive electrostatic interaction between the biopolymers, which
413 causes them to associate with each other and form small microgels that trap the bioactive-loaded lipid droplets
414 inside. Fourth, these microgels are stabilized by either hardening them or by forming a coating around them.
415 Hardening can be achieved by altering the temperature, or by adding an agent that cross-links one or both of the

416 biopolymers inside the microgels (Donbrow, 1991). Coating can be achieved by adding an oppositely charged
417 biopolymer that adsorbs to the surfaces of the microgels due to electrostatic attraction. The combination of gelatin
418 and gum Arabic is frequently used to form complex coacervates (Jincheng, Xiaoyu, & Sihao, 2010). Complex
419 coacervation has been used for encapsulation of tuna oil using gelatin–sodium hexametaphosphate {Wang, 2014
420 #5}, fish oil using cress seed mucilage-chitosan {Kavousi, 2017 #6}, palm oil using chitosan-xanthan and chitosan-
421 pectin {Rutz, 2017 #7} and sulforaphane using gelatin-gum Arabic and gelatin-pectin complexes {García-Saldaña,
422 2016 #8}.

423 *3.2.2. Cold gelation*

424 Cold gelation can be used to produce protein-based gels at ambient temperatures using a multi-step process
425 {Alting, 2002 #13}. First, a solution of globular proteins is heated above its thermal denaturation temperature under
426 controlled pH and ionic strength to induce protein unfolding and filament formation. Second, the heat-denatured
427 protein solution is cooled to ambient temperature and mixed with the bioactive agents. Third, the heat-denatured
428 proteins are made to associate with each other and form a hydrogel by adjusting the pH or adding salt to reduce the
429 electrostatic repulsion between the proteins (Fig. 4).

430 Various kinds of globular proteins, including soy (Zhang, et al., 2012) and whey proteins (Sadeghi, Madadlou,
431 & Yarmand, 2014) have been used to develop encapsulation systems using the cold gelation method. For soy
432 proteins, an increase in calcium concentration and a decrease in pH led to the formation of nanoparticles (Zhang, et
433 al., 2012). {Beaulieu, 2002 #3} produced protein aggregates by heat treatment followed by cold gelation by
434 gradually lowering the pH and investigated importance of the net electric charge of the aggregates during gel
435 formation. With decreasing pH, disulfide cross-links between modified aggregates were not formed and the gels
436 displayed both syneresis and spontaneous gel fracture.

437 *3.2.3. Thermal gelation*

438 Heating of an aqueous solution of globular proteins above their thermal denaturation temperature induces
439 protein unfolding and possible aggregation {Wang, 1991 #14}. Protein aggregation tends to occur due to the
440 increase in the surface-hydrophobicity of the proteins after thermal denaturation, and may be further stabilized by
441 disulfide bond formation. At high protein concentrations, a macroscopic hydrogel is formed, but at lower protein
442 concentrations nanoscopic or microscopic hydrogels can be formed. The characteristics of the hydrogels formed

443 also depend on protein type, protein concentration, pH, and ionic composition of the dispersion (Gaonkar, Vasisht,
444 Khare, & Sobel, 2014).

445 Madadlou et al (2014) encapsulated caffeine via a microemulsification/heat gelation method. Enzymatically
446 cross-linked whey proteins were heated at 90 °C for 15 min to fabricate nanocarriers. The results showed that
447 enzyme treatment led to smaller and highly monodisperse particles, with a higher degree of sphericity in comparison
448 to non-treated samples. Due to the high applied temperature, this technique is not suggested for heat sensitive
449 compounds.

450 *3.2.4. Spray drying*

451 Spray drying has been used for the preparation of protein-based delivery systems, for example using soy
452 proteins (Tang & Li, 2013) and whey proteins (Pérez-Masiá, et al., 2015). The selection of appropriate wall
453 materials is mainly based on their surface activity, solubility, low viscosity at high solid content, and glass forming
454 properties {Ré, 1998 #15}. Grafting fatty acid chains to proteins by acylation was reported to enhance the surface
455 hydrophobicity, the amphiphilic characteristics, the solubility and the structural properties of the proteins, improving
456 their encapsulating during spray drying (Nesterenko, Alric, Silvestre, & Durrieu, 2012).

457 The spray drying process usually leads to the formation of micron-sized carriers. Recently, nano-spray dryers
458 have been developed, which enable the fabrication of submicron particles by effective fluid breakdown combined
459 with highly efficient particle collectors. This technology utilizes a vibrating mesh works with ultrasonic frequency
460 that ejects millions of precisely nano-sized droplets. Dried particles are then collected by an electrostatic particle
461 collector which is size independent in contrast to cyclones (Fathi, et al., 2014). Haggag and Faheem {Haggag, 2015
462 #19} investigated application of nano spray drying for drying and formulation of therapeutic peptides and proteins.

463 *3.2.5. Electro-hydrodynamic processes*

464 Electro-hydrodynamic processes are based on the use of electrical charges to produce very thin fibers or fine
465 particles {Drosou, 2016 #16}. Electro-spinning and electro-spraying are used for production of nanofibers and
466 nanoparticles by applying high intensity electric fields (kV/cm range) to biopolymer solutions. The principles of
467 electro-spinning and electro-spraying have been reviewed in previous papers (Fathi, 2015; Fathi, et al., 2014). Due
468 to their relatively high surface charge characteristics, some proteins have been shown to be particularly suitable for

469 formation of nanoparticles using this method, including soy protein (Vega-Lugo & Lim, 2008; Zhao, et al., 2010)
470 and gelatin (Mindru, Mindru, Malutant, & Tura, 2007; Okutan, Terzi, & Altay, 2014). However, the electro-
471 spinning properties of proteins can be further enhanced by the addition of other polymers, such as polyethylene
472 oxide and polyvinyl alcohol (Tang, Ozcam, Stout, & Khan, 2012; Vega-Lugo & Lim, 2012; Woerdeman, Shenoy, &
473 Breger, 2007).

474 *3.2.6. Antisolvent precipitation methods*

475 Liquid–liquid dispersion, also known as antisolvent precipitation, techniques are commonly used to produce
476 protein nanoparticles {Joye, 2013 #174;Patel, 2014 #192}. In this process, proteins that are soluble in organic
477 solvent (e.g. zein) are first dissolved in an ethanol-water solution, which is then dispersed into an antisolvent
478 solution (water) with continuous agitation. As a result, the proteins become insoluble and precipitate to form
479 nanoparticles because the ethanol concentration in the solution drops below the solubilization limit (Wu, et al.,
480 2012b). Zein nanoparticles can be formed using this method when the fabrication conditions are optimized, such as
481 zein concentration, alcohol level, alcohol type, and mixing conditions (Zhong & Jin, 2009). A positive correlation
482 has been reported between the number of sulfhydryl (-SH) groups in zein and the size of the nanoparticles formed in
483 different alcohol solutions (Chen, Ye, & Liu, 2013). Moreover, the increase in concentration of alcoholic solution
484 induces the exposure of internal sulfhydryl groups to the exterior of the unfolded proteins, leading to the formation
485 of disulfide bonds and insoluble aggregates. The size of the protein particles formed decreased as the ethanol
486 content increased from 70% to 90%, and then increased at higher alcohol levels, which was attributed to a structural
487 change in the proteins (Kim & Xu, 2008).

488 **4. Release mechanisms**

489 Towards the development of controlled and tailored delivery systems, understanding the release mechanism
490 plays an important role for better protection of the bioactives, as well as for modeling and optimization of the release
491 phenomenon {Wise, 2000 #17}. Depending on the composition of the protein, the nature of the bioactive
492 molecules, the release media, the loading amount, and the particle geometry, different release mechanisms can
493 occur. Four main mechanisms governing the release phenomena from protein nanoparticles are outline below:

494 (i) *Diffusion*: For non-biodegradable biopolymers, the bioactive molecules simply diffuse out of the intact
495 nanoparticles and into the surrounding medium. Diffusion can occur through water-filled pores (Fig. 5. A),

496 through the homogeneous matrix (Fig. 5. B), or from an internal reservoir through an external shell (Fig. 5. C).
497 The mass transfer rate depends on the size and geometry of the carrier, on the solubility of the bioactive in the
498 matrix, as well as on its diffusion coefficient through the matrix. The diffusion coefficient is affected by
499 different particle and environmental parameters, such as tortuosity (the lower the tortuosity, the higher the
500 diffusion coefficient), porosity (the higher the porosity, the higher the diffusion coefficient) and temperature
501 (the higher the temperature, the higher the diffusion coefficient).

502 (ii) *Erosion*: The bioactive component is released into the media due to erosion processes taking place at the
503 nanoparticle surface (heterogeneous erosion; Fig 5. D) or in the bulk volume of the nanoparticle (homogenous
504 erosion; Fig 5. E). Erosion could be induced by chemical or enzymatic processes. In surface erosion, the
505 biopolymer is eroded at the external boundary, which causes a gradual size reduction. In contrast, in bulk
506 erosion, the size of the particles remain almost constant, and the external fluid penetrates into the system by
507 breaking the physical or chemical bonds (Zhang, Yang, Chow, & Wang, 2003). The erosion rate depends upon
508 the biopolymer molecular mass (the lower the molecular mass, the higher the erosion), physicochemical
509 stability, size (the smaller the size, the higher the erosion) and the release media (Chirico, Dalmoro, Lamberti,
510 Russo, & Titomanlio, 2007).

511 (iii) *Swelling/shrinkage*: In the swelling-induced release mechanism, the bioactive is initially trapped within a
512 nanoparticle that has a small pore size (compared to the dimensions of the bioactive). The system conditions
513 are then changes (such as water activity, temperature, pH, or ionic strength), which causes the nanoparticle to
514 swell, which increases the pore size, and releases the bioactive (Fig. 5. F). In contrast, in the shrinkage
515 mechanism, the bioactive component, which is loaded into the nanocarriers by initially swelling them, is
516 released by changing the solution conditions to induce the shrinkage and the payload release (Fig. 5. G) (Arifin,
517 Lee, & Wang, 2006; Jones, 2009).

518 (iv) *Fragmentation*: The active component is released into the media due to the physical disruption of the
519 nanoparticles, which is either fragmented or fractured by applying shear or compression forces during
520 processing, or in the mouth and gastrointestinal media (Fig. 5. H) (Jones, 2009).

521 It should be noted that, in all the above mechanisms, diffusion is always involved. Mathematical modeling of
522 bioactive release from nanoparticles can be used to facilitate the design of encapsulation and delivery systems with
523 desired release profiles {Fathi, 2014 #18}. To select an appropriate model of release, preliminary knowledge of the

524 release mechanism is required. The size of the nanoparticles, the porosity, the pore size distribution, the
525 concentration, and solubility of the bioactive molecules in the matrix and in the release media, as well as the
526 effective diffusion coefficients are needed to understand the release mechanism. Such data are sometimes available
527 for drug delivery systems; in contrast, for food systems only a few data have been reported in recent years (Fathi &
528 Varshosaz, 2013; Fathi, Varshosaz, Mohebbi, & Shahidi, 2013). Therefore, simplified empirical models have been
529 proposed to describe the release according to the different mechanisms (Table. 3) (Cooney, 1972). Mechanistic
530 models were also developed for diffusion- (Martinez, et al., 2013), erosion- (Sackett & Narasimhan, 2011; Tzur-
531 Balter, Young, Bonanno-Young, & Segal, 2013) and swelling- (Kaunisto, Marucci, Borgquist, & Axelsson, 2011)
532 controlled release. Recently novel modeling systems such as cellular automata (Fathi, Mohebbi, Varshosaz, &
533 Shahidi, 2013; Laaksonen, Hirvonen, & Laaksonen, 2009) and Monte Carlo (Vlugt-Wensink, et al., 2006) have been
534 adapted to predict the release of bioactives from nanoparticles. However, more studies are needed for a better
535 modeling of the release of food bioactives from polymeric delivery systems in different media (food systems and
536 gastrointestinal fluids).

537 **5. Delivery system design**

538 Protein-based delivery systems can be fabricated using a wide variety of different kinds of animal and plant
539 proteins, and using a range of different production methods. A manufacturer may therefore have to select the most
540 appropriate one for a particular application. Several factors affect the selection of appropriate ingredients and
541 encapsulation methods: (i) *compatibility* – is the nanocarrier chemically or physically compatible with the food
542 components? (ii) *stability* – is the nanocarrier stable under processing, storage or consumption of food? (iii) *release*
543 *mechanism and condition* – which kind of release mechanism is expected and under which condition release is
544 supposed to occur? (iv) *biodegradability* – is the protein or protein-based nanocarrier degraded in the body? (v)
545 *economic feasibility* – can the nanocarrier be economically and reliably made on a large enough scale for practical
546 applications.

547

548 **6. Conclusions and future directions for research**

549 The field of protein-based delivery systems is growing rapidly because of the perceived benefits of these
550 natural polymers for encapsulating, protecting, and releasing bioactive agents. This article provided an overview of
551 the different animal, plant, and recombinant proteins that can be used to develop nanoscale delivery systems for
552 bioactive components suitable for utilization within the food industry. It also described a number of particle
553 fabrication methods that have been developed to create these delivery systems, and highlighted some of the
554 advantages and disadvantages of each method. Future research should determine whether these methods are
555 economically feasible for large-scale production and to establish whether they can function under the harsh
556 environmental conditions present in many food products. Moreover, research is also needed to understand how they
557 behaved within the human gastrointestinal tract after ingestion.

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952 Food-grade nanoparticles for encapsulation, protection and delivery of curcumin: comparison of lipid,
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957 Table 1. Physicochemical properties of some proteins commonly used for the delivery of food bioactives

Protein	Molecular mass (kDa)	IP	Properties	Reference
Collagen	0.01-20000	5	High temperature stability	(Bourne, 2014; Ramachandran, 2013)
Gelatin	20-250	Acidic gelatin= 5 Basic gelatin=9	Soluble in hot water; high temperature stability	(Domb, Kost, & Wiseman, 1998)
Casein	Alpha-casein=23 Beta-casein=24 Kappa-casein=19	4.6-4.8	Poorly soluble in water; micellar; high temperature stability	(Belitz & Grosch, 2013)
Beta-lactoglobulin	18.2	5.2-5.4	Water soluble; globular; acid resistant	(Belitz, et al., 2013; Zehren, 1954)
Alpha-lactalbumin	14	4.4	Water soluble; globular	(Belitz, et al., 2013; Varnam, 2012)
Bovine serum albumin	65	5.5	Water soluble; globular; antioxidant	(Belitz, et al., 2013; Nakai & Modler, 1996)
Albumin	0.01-20000	Ovalbumin=4.6-4.8 Bovin serum albumin=4.7 Human serum albumin=4.7	Water soluble	(Nakai, et al., 1996)
Silk fibroin	60-150	2.1	Water insoluble;	(Hawley &

			Fibrillar; high mechanical properties;	Johnson, 1930; Salamone, 1996)
Zein	Zein=45 Alpha zein= 21-25 Beta zein=17-18 Delta zein =9-12 Gamma zein=26-29	6-7	Water insoluble; alcohol soluble	(Larkins & Vasil, 2013)
Soy protein	7S=150-190 11S=320-360	4.5	Water soluble; composed of albumins and globulins	(Belgacem & Gandini, 2011)
Pea protein	320-380	4.3-4.5	Water soluble; high emulsifier ability	(Barac, et al., 2010; Tarté, 2009)

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959 Table 2. Production methods of protein-based nanoencapsulation systems

Nanoencapsulation methods	Advantage and disadvantage	Reference
Coacervation	Can be implemented in large scale production; applicable for charged biopolymers	(Ilyasoglu & El, 2014)
Cold induced gelation	Is suitable for temperature-sensitive compounds; can be implemented for large scale production	(O'Neill, Egan, Jacquier, O'Sullivan, & O'Riordan, 2014; Zhang, Liang, Tian, Chen, & Subirade, 2012)
Heat induce gelation	It is not suggested for heat sensitive compounds; can be implemented for large scale production	(Gaonkar, Vasisht, Khare, & Sobel, 2014)
Spray drying	Different kind of proteins can be used; it is not suggested for heat sensible compounds; can be implemented in large scale production	(Pérez-Masiá, et al., 2015)
Electrohydrodynamic process	High mechanical properties of nanocarriers; dry sample is obtained and solvent evaporation is not needed; not yet implemented in large scale production	(Kriegel, Arrechi, Kit, McClements, & Weiss, 2008)

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961 Table 3. Kinetic models for analysis of encapsulant release from nanocarriers.

Model	Equation	Model parameter	Reference
Weibull	$C = C_0\left[1 - e^{-\frac{(t-T)^b}{a}}\right]$	a, time dependent parameter; b, shape parameter	(Dash, Murthy, Nath, & Chowdhury, 2010)
Higuchi	$C = kt^{0.5}$	k, kinetic constant	(Barzegar-Jalali., et al., 2008)
Zero order	$C = kt$	k, kinetic constant	(Fathi, Varshosaz, Mohebbi, & Shahidi, 2013)
First order	$C = [1 - \exp(-kt)] \times 100$	k, kinetic constant	(Fathi, et al., 2013)
Rigter-Peppas	$C = kt^n$	k, kinetic constant; n, release exponent used to characterize release mechanism (for spherical carries n $\leq$ 0.43 is controlled by Fickian diffusion mechanism, and n $\geq$ 0.85 is commanded for dissolution phenomenon, and 0.43< n $\leq$ 85 is governed by combination of two mechanisms)	(Dash, et al., 2010)
Reciprocal powered time	$(\frac{1}{C} - 1) = \frac{m}{t^b}$	b, a model parameter	(Mohammadi, et al., 2010)

		(describing the shape); m, parameter (related to the time required for 50% release, t50%)	
Linear probability	$C = C_0 + kt$	k, kinetic constant	(Barzegar-Jalali., et al., 2008)
Log – probability	$C = C_0 + k \ln t$	k, kinetic constant	(Barzegar-Jalali., et al., 2008)
Hixson-Crowell	$C_0 - \sqrt[3]{1-C} = kt$	k, kinetic constant incorporating surface to volume relation	(Costa & Sousa Lobo, 2001)

962 C_0, initial concentration; C, concentration in time t

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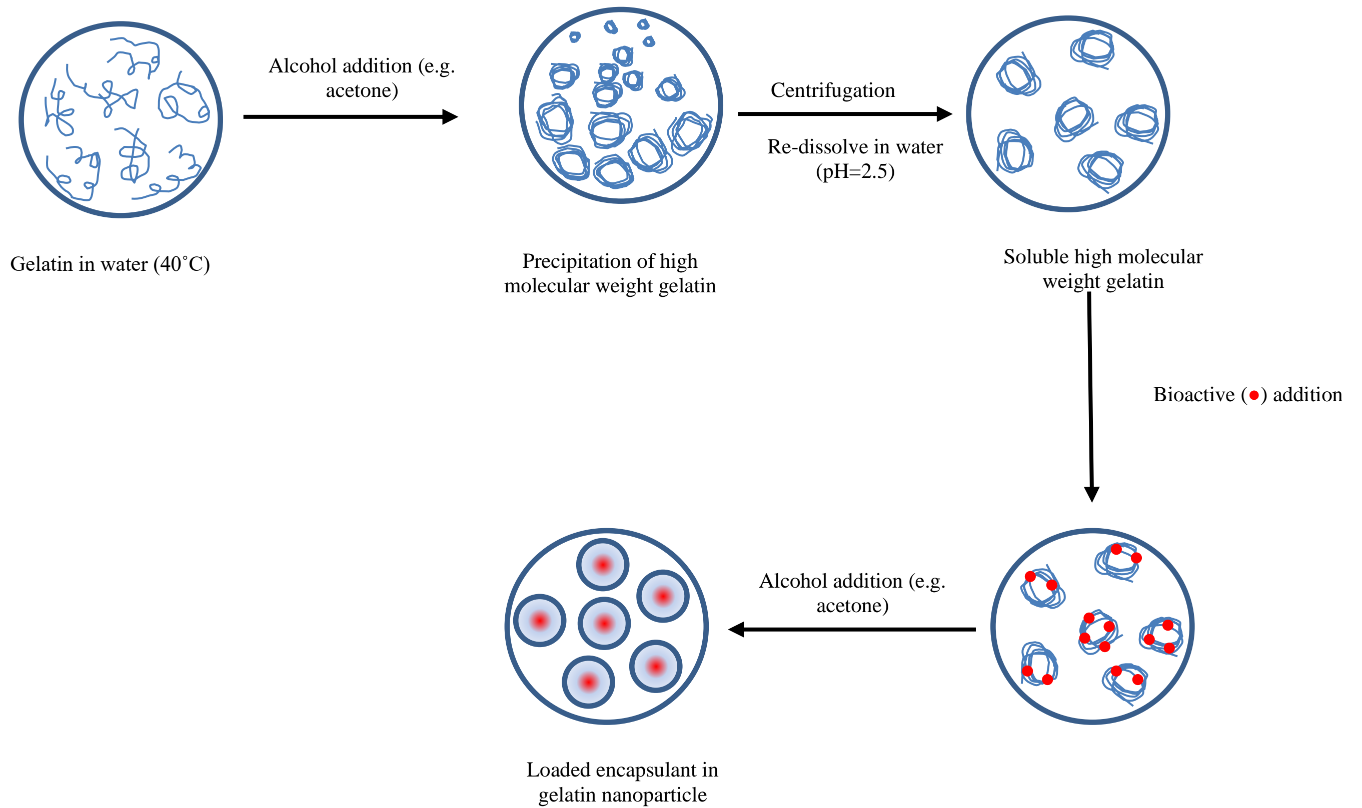

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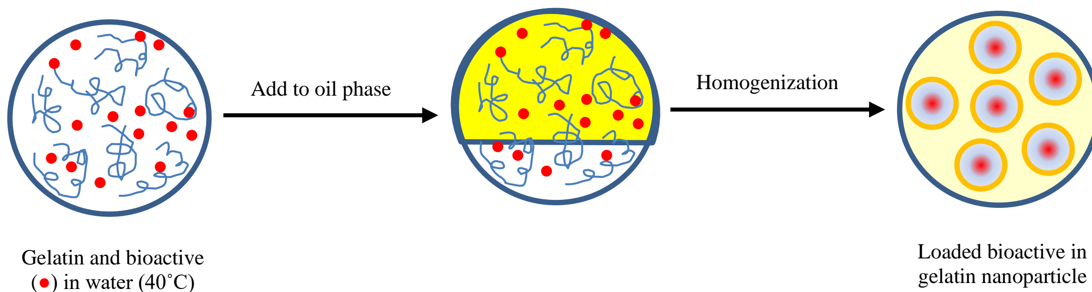

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968 Fig. 1. Production of bioactive-loaded gelatin nanoparticles using the desolvation/coacervation (A) and emulsion (B)

969 methods.

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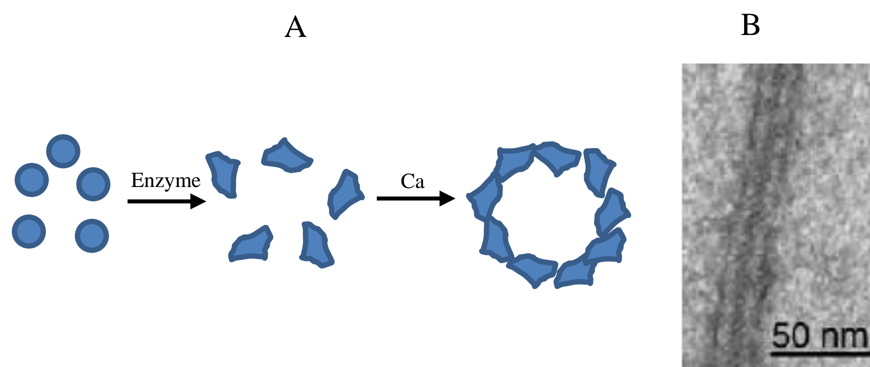
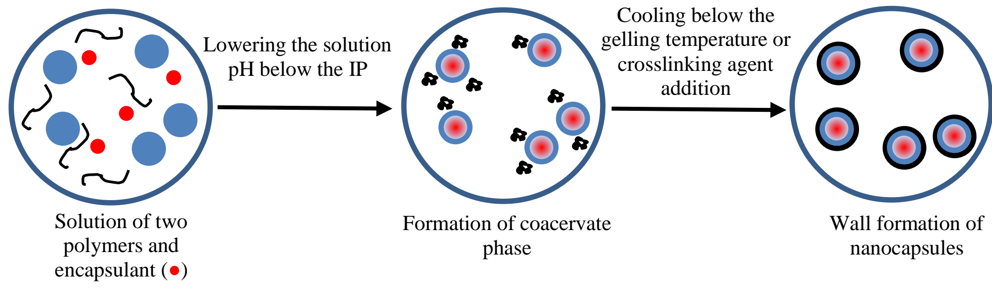


Fig. 2. Schematic representation of the self-assembly enzymatic hydrolysis of α-LA in the presence of Ca^{+2} into nanotubes (A). Transmission electron microscopy of α-LA nanotube (B) (Graveland-Bikker & de Kruif, 2006) (with permission).



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977 Figure 3. Complex coacervation of proteins and carbohydrates.

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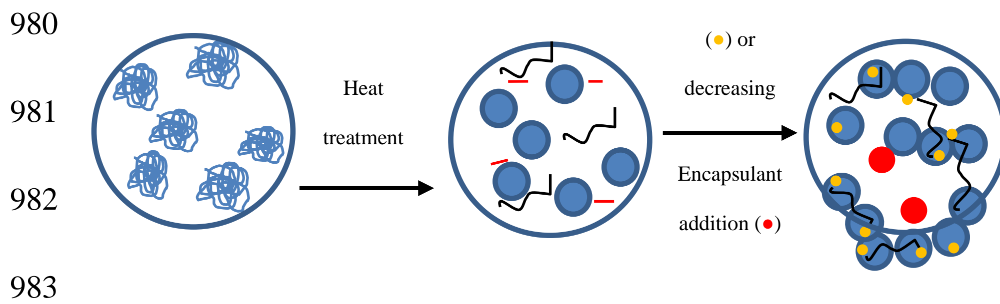

984 Fig. 4. Schematic representation of cold gelation of proteins.

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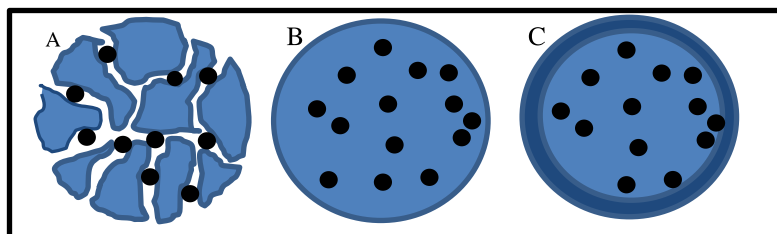

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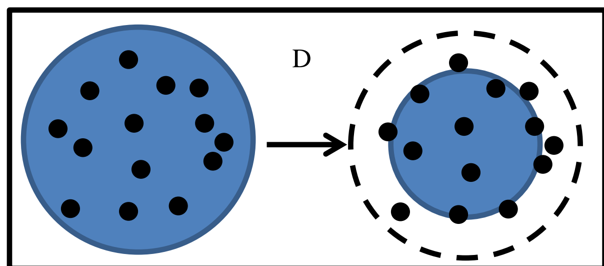

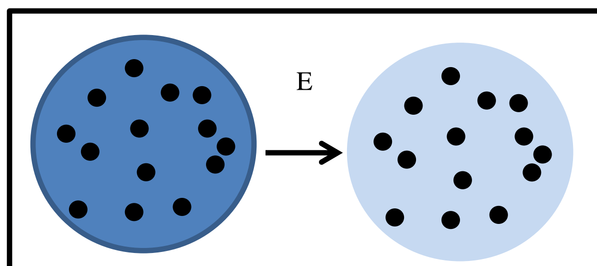

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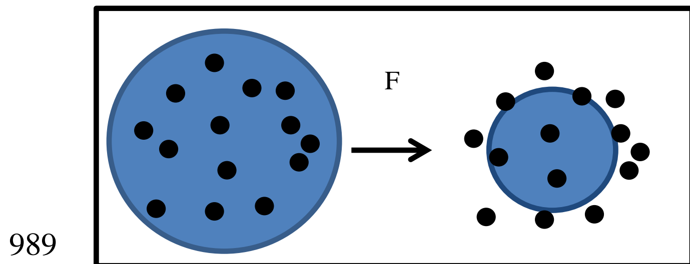

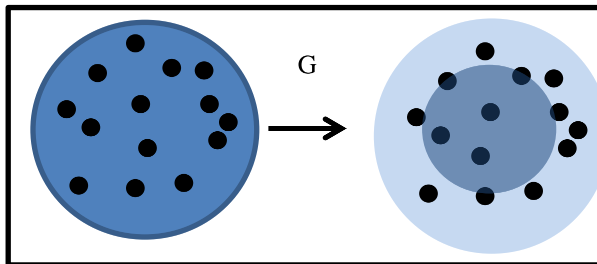

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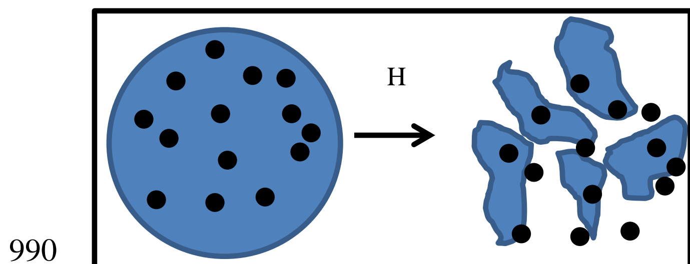

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991 Fig. 5. Release mechanism from protein nanocarriers; A, water-filled pores; B, homogeneous matrix diffusion; C,
992 reservoir matrix diffusion; D, heterogeneous erosion; E, homogenous erosion; F, shrinkage; G swelling; H,
993 fragmentation.

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