Chapter 7
β-Lactoglobulin-Based Nano and Microparticulate Systems for the Protection and Delivery of Bioactives

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Abstract The new paradigms in human nutrition and the ever-increasing consumer demand for safe and healthy food products have encouraged research in functional foods and nutraceuticals as pharmaceutical surrogates. Food proteins are abundant and from renewable sources, with functional groups conferring interesting structural and functional properties. Their ability to bind small ligands and to form aggregates and electrostatic complexes with other food macromolecules provides numerous applications for oral delivery technology. The current review focuses on the major milk protein β-lactoglobulin, its techno-functional properties and its applications in the formulation of nano- and micro-sized oral delivery platforms.

7.1 Introduction

Active compounds such as probiotics, bioactive peptides, antioxidants and vitamins with physiological benefits beyond basic nutritional functions such as reducing the risk of chronic disease, are often referred to as nutraceuticals. In Canada, they

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Y.H. Roos and Y.D. Livney (eds.), *Engineering Foods for Bioactives Stability and Delivery*, Food Engineering Series,
DOI 10.1007/978-1-4939-6595-3_7

fall under the regulation of natural health products (NHPs), which went into effect on January 1, 2004 (Canada 2013). The market for NHPs is increasing rapidly worldwide. The market value is estimated at around US $117 billion from the three most important sources alone—the Unites States, Japan and Western Europe (Núñez Sellés 2011). This increases to between $300 and $400 billion when other areas such as Eastern Europe, Latin America, Asia or Africa are considered.

The development of the ideal oral system for the protection and delivery of NHPs has become a focus of tremendous research worldwide, particularly in the food and pharmaceutical sciences. The popularity of oral delivery systems has soared, and sales were already expected to reach over $67 billion a few years ago (Kim and Pack 2006; Ranade and Cannon 2011). The designation 'oral delivery system' usually implies 'controlled delivery' of bioactive compounds, which involves the efficiency of delivery and the enhancement of solubility, absorption, bioavailability, safety and duration of action (Ranade and Cannon 2011). In addition to increasing patient comfort and compliance, oral delivery systems can achieve sustained, controlled and site-specific release of bioactives (Santus and Baker 2003; Kim and Pack 2006; Ranade and Cannon 2011; Murray 2011). Consequently, from a pharmaceutical point of view, serum concentrations are less prone to fluctuations, and toxicity and dosing frequency are reduced (Ranade and Cannon 2011). In the food industry, delivery systems are used as carriers for functional ingredients, protecting those ingredients from hostile environmental factors including heat, light, oxygen, high pressure and shear forces during processing, storage and utilization, until the intact bioactive ingredient is delivered to the target site; the list of advantages is not exhaustive. The release rate of the bioactive molecule can then be modulated via the control of conditions including temperature, ionic strength, pH and digestive enzymes (Weiss et al. 2006).

The release kinetics from the oral delivery system rely on the chemical composition and/or the structure of the carrier system. In general, mechanisms of release such as diffusion result from physical (erosion) or chemical decomposition (degradation); swelling and osmotic pressure lead to an expansion of the carrier system, thus expelling the bioactive out of the carrier matrix (Ranade and Cannon 2011; Murray 2011). The chemical composition and structure of the oral delivery system are of utmost importance in achieving effective release of bioactives. With the size of oral delivery systems decreasing dramatically, concerns have been raised regarding the toxicity of such carriers and the resulting degradation products. Indeed, reduced size may lead to an increase in toxicity, given that small sized carriers might reach and accumulate in regions within cells or tissue inaccessible to macroscopic particles of the same composition. In fact, the size of oral delivery systems has decreased from capsules, tablets or powder forms to microparticles, nanoparticles and nanocomplexes. Microparticles are less than about 1000 μm while nanoparticles and nanocomplexes are less than 100 nm in size (Chen et al. 2006; Weiss et al. 2006). Therefore, biopolymers which are biocompatible and biodegradable with negligible to null toxicity are increasingly used to fabricate oral delivery systems. Milk whey protein fits well with these criteria, and thus whey protein-based delivery systems have been the focus of countless peer-reviewed

articles (Beaulieu et al. 2002; Chen and Subirade 2005; Chen et al. 2006; Chen and Subirade 2006; Hebrard et al. 2006; Hebrard et al. 2009; Hebrard et al. 2010; Hebrard et al. 2013). However, whey proteins are composed of diverse proteins with different structures, properties and functions, making it difficult to distinguish the role and degree of participation of each protein in the formation of the carrier system (O'Regan et al. 2009). However, most functional properties of whey isolates, including aggregates and gel formation, are attributed to one major protein, β-lactoglobulin (βlg) (Boland 2011; Nicolai et al. 2011). The present review therefore concentrates on the major whey protein β-lactoglobulin (βlg) and its functionality as a micro and nano-based oral delivery system.

7.2 Structure and Functionality of βlg

The structure and amino acid composition of βlg have been the focus of extensive research (Oliveira et al. 2001; Kontopidis et al. 2004; Loch et al. 2011; Sawyer 2013). Thus, only the structural characteristics and physicochemical properties related to bioactive protection and delivery are detailed in the present review.

Well defined crystal structures indicate that βlg is a globular protein of the lipocalin family, with a molecular weight of 18.3 kDa and an isoelectric point (p*I*) of 5.3 (Oliveira et al. 2001; Boland 2011; Sawyer 2013). The two main genetic variants of βlg, A and B, differ from a mutation occurring at amino acid sequence position 64 ($Asp_A \rightarrow Gly_B$) and 118 ($Val_A \rightarrow Ala_B$). The overall conformation of the molecule remains fairly the same, although the substitutions lead to dissimilarities in properties such as heat stability and resistance to pressure denaturation (Botelho et al. 2000; Oliveira et al. 2001). The information presented in the current review will refer to βlg variant B.

The protein is constituted of 162 amino acids, including all 20 amino acids in relative amounts that make it exceptional and valuable nutritionally. In fact, compared to theoretical common values computed for proteins, βlg comprises about 17 % more essential amino acids (Mehra and O'Kennedy 2009). The tertiary structure of βlg is composed of 8 % of α-helix, 45 % of β -sheet and 47 % of random coil (Loch et al. 2011; Oliveira et al. 2001; Sawyer 2013). βlg has an overall radius of 2 nm, with almost 90 % of its mass within 1 nm of the surface and nearly 60 % within 0.5 nm (Liang and Subirade 2012). The structure of the monomer of βlg is composed of nine strands of antiparallel β-sheets (strands A to I), eight (strands A to H) of which wrap around to form a flattened, conical barrel, also called the central calyx. The cylindrical-shaped calyx has a length of 15 Å, with hydrophobic walls composed of two sheets made of strands A–D and strands E–H. Strand A (residues 16–27) participates in both sheets due to its 90º bend at its midpoint (Ser21), while the ninth strand (I) extends the EFGHA sheet. The neighboring strands within the sheets are connected via a loop (Uhrínová et al. 2000). The dimer interface is formed in part by strand I and the loop connecting strand A to B. Strand A is preceded by a three-turn α-helix while another one lies in the A–B loop. The structure of βlg

contains two disulfide bridges consisting of Cys66–Cys160, connecting the C–D loop to the carboxyl-terminal region, and Cys106–Cys119 links strands G and H. An additional Cys at position 121 is buried and thus remains free. At pH values from 5 to about 7, including that of the milk (∼pH 6.6), βlg exists as a dimer in solution; below pH 3 and above 7, the monomeric conformation predominates (Uhrínová et al. 2000; Sawyer 2013). Upon modification of the pH, βlg undergoes different structural transitions categorized into distinct classes (M, Q, N and R), and their interrelation is as follows: M ↔ Q ↔ N ↔ R (Sakurai and Goto 2007).

Reports indicate that moderate to considerable pH-induced structural transitions of βlg occur between pH 2.5 and 8, while above pH 9, βlg undergoes significant and irreversible structural modification, also called base-induced unfolding of the protein (Uhrínová et al. 2000; Taulier and Chalikian 2001; Sakurai and Goto 2007). Below pH 2, the volume and compressibility of the protein decrease, providing βlg with a more compact structure (Taulier and Chalikian 2001). It is believed that further structural transition continues at pH values less than 1.0, even though no report exists at the moment. Between pH 2.5 and 8, the overall conformation of the protein is conserved extraordinarily well, despite significant structural changes.

The dimer-to-monomer transition occurring between pH 4.5 and 2.5 is the acid-induced dissociation of the dimeric βlg into monomers. It includes the βlg transition from the monomeric (M) to the acidic form (Q) around pH 3, where the protein is believed to dimerize (Taulier and Chalikian 2001; Sakurai and Goto 2007). This transition triggers a different orientation of the α-helix, which affects only the surface electrostatic properties of βlg. The native dimeric (N) to the acidic (Q) form transition occurs between pH 6.0 and 4.5. This transition is accompanied with a change in the compactness of the protein translated into a slight expansion of the hydrodynamic volume of βlg (Taulier and Chalikian 2001; Sakurai and Goto 2007). It also includes the pH 5 transition (dimer to octamer transition from pH 3.9 to 5), at which βlg undergoes an octamerization without significantly affecting its secondary structure. Above pH 5, the dimerization of βlg is due to the electrostatic interactions between Asp130 and Glu134 of one monomer with corresponding lysyl residues of other monomers. The Tanford (N–R) transition occurs between pH 7 and 8 (Taulier and Chalikian 2001; Sakurai and Goto 2007). This transition involves a conformational change of the E–F loop (residues 85–90), probably due to the cleavage of hydrogen bonds between the F and G strands, as represented in Fig. 7.1 (Sakurai and Goto 2006; Sakurai and Goto 2007). Finally, there is a transition at pH 9.0 or above where βlg undergoes an irreversible base-induced unfolding (Taulier and Chalikian 2001). These structural transitions are important for understanding the functional properties of the protein.

7.3 Functionality of βlg

The physiological role of βlg, albeit not fully understood, seems to be intimately related to its amino acid composition and tertiary structure. The health benefits of βlg and derived peptides go far beyond its undeniable nutritional value and well

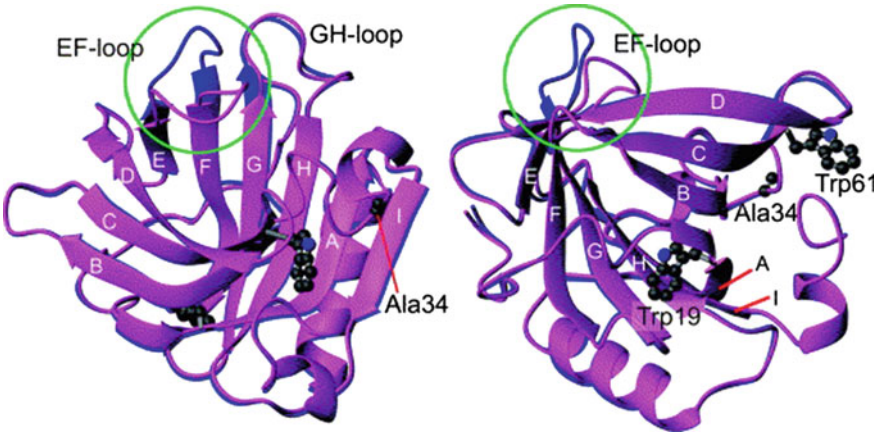

Fig. 7.1 Structures of βlg displaying the E–F loop in the closed (*left*) and open (*right*) conformation in the *green* circle. The conical β-barrel is formed by two sheets consisting of β-strands *A–D* and strands *E–H* [adapted from Sakurai and Goto (2006), Sakurai and Goto (2006)]

justify its use in functional food ingredients and nutraceuticals. Their effects on a number of disease conditions have been reviewed elsewhere (Mehra and O'Kennedy 2009; Morris and FitzGerald 2009). Examples of beneficial activities on human health include their role as hypotensive, anticancer, immunomodulatory, opioid agonist, mineral binding, antimicrobial, gut health-enhancing, hypocholesterolemic, insulinotropic and psychomodulatory agents.

Functional advantages of βlg reside in its capacity for gel formation, foaming and emulsion stabilization, all of which find numerous applications in the food industry (Chen et al. 2006; Tunick 2009; Nicolai et al. 2011). Gelation remains one of the most important techno-functional properties of βlg and is traditionally achieved though thermal treatment. The sequential gelation steps consist of the unfolding of polypeptide chains with concomitant exposure of initially buried hydrophobic amino acid residues and subsequent self-aggregation of protein molecules into a three-dimensional network that entraps water by capillary forces (Chen et al. 2006). Contributing forces are hydrophobic effects, van der Waals interactions, hydrogen bonding and covalent interactions, of which the impacts can be determined via the use of destabilizing agents. As one example, urea can be used to block the formation of the hydrogen bonds, sodium dodecyl sulfate (SDS) to block the formation of the hydrophobic interactions, and 2-mercaptoethanol to block the formation of the disulfide bridge (Remondetto and Subirade 2003). In the absence of salt, βlg forms transparent 'fine-stranded' gels at extreme pH values away from its p*I*, and opaque 'particulate' gels near its p*I*. Salt has been used to induce cold-set gels made by prior heat denaturation of βlg (Remondetto and Subirade 2003; Chen et al. 2006).

The solubility of βlg is greatly enhanced in the presence of salt due its surface charge distribution at neutral pH, thus explaining the harvesting of the protein by dialysis or precipitation upon salting out and further growing of X-ray crystal

structures (Creamer et al. 2011; Sawyer 2013). The structure of βlg justifies its classification as a member of the lipocalin family and calycin subclass, which is naturally involved in the transportation of small hydrophobic bioactives (Sakurai et al. 2009). Reports indicate that βlg binds retinol, triglycerides and long chain fatty acids such as palmitic acid, resulting in enhanced intestinal uptake of these ligands. βlg might also have an important role in carrying implicated ligands in food systems and pharmaceutical preparations, as well as in digestion, absorption and metabolism of some of implicated ligands in neonates (Mehra and O'Kennedy 2009). However, the sequence similarity of βlg with glycodelin, an important protein for fetal development expressed in the endometrium during the first trimester of human pregnancy, suggests further important biological functions (Sakurai and Goto 2006; Sawyer 2013). This is confirmed by the absence of βlg from human and rodent milk and its presence in the whey of ruminants' milk, including cow's milk, thus making its true function still elusive (Bonnaillie and Tomasula 2009; Sawyer 2013). However, there is a general consensus about its role as ligands transporter. Intensive literature exists on the large selection of ligands that bind to βlg (Kontopidis et al. 2004; Creamer et al. 2011; Sawyer 2013). This ligand binding property is mainly ascribed to the presence of the central calyx, which plays the role of a receptacle for hydrophobic and amphiphilic small molecules, consequently forming βlg–ligand complexes (Liang et al. 2008; Liang and Subirade 2010; Diarrassouba et al. 2013).

7.4 Structural Basis for the Formation of the βlg–Ligand Complexes

The folding of the two β-sheets (A–D and E–H) into a central cavity paneled with hydrophobic amino acids resembles a barrel with the EF loop (residues 85–90) acting as the gate (Sakurai et al. 2009). The EF loop folds over the entrance of the calyx to form a closed conformation at pH values lower than 6.5. At pH values above 7, it adopts an open conformation which exposes the interior of the calyx to what? (Qin et al. 1998). This conformational flexibility is attributed to the Tanford transition, which is accompanied by the deprotonation of the carboxyl group of Glu 89, located on the EF loop. Glu 89 that has an anomalous p*Ka* of 7.3 is normally buried and protonated in the 'closed conformation' at acidic pH. At pH values above 7, Glu 89 is exposed to what? and deprotonated, thus triggering the opening of the EF loop. This consequently offers access to the central calyx (Sakurai et al. 2009). The pH-controlled flipping of the EF loop seems to be crucial for the physiological significance of βlg, since in the closed conformation bound ligands might be protected in the acidic stomach in order to be further released within the intestines at higher pH values (Kontopidis et al. 2004; Sakurai and Goto 2007; Sawyer 2013).

A number of methods have been used to study the binding of the ligands to βlg (Collini et al. 2003; Ragona et al. 2003; Riihimäki et al. 2008; Yang et al. 2009; Liang and Subirade 2010; Loch et al. 2011; Diarrassouba et al. 2013). However, spectroscopic methods are among the most user-friendly, rapid, accurate and less cumbersome techniques used to investigate the protein–ligand interaction. Fluorescence spectroscopy is preferred for studying the binding stoichiometric of the βlg–ligand complex, while circular dichroism is interesting for studying the influence of the ligand binding on the secondary and tertiary structures of the protein.

Fluorescence spectroscopy, and in particular fluorescence quenching, is commonly used to characterize ligand binding to proteins. Fluorescence quenching refers to any process that reduces the fluorescence intensity of a sample caused by processes such as the inner-filter effect, energy transfer, ground state complex formation and collisional processes (Zhao et al. 2006; Rouabhia et al. 2007). The inner-filter effect occurs when the fluorescence emission of the fluorophore is affected by the presence of an absorbing substance that absorbs the radiation going towards (excitation) or emanating from (emission) the fluorophore (van de Weert 2010). The resulting fluorescence quenching will thus result from the reduction of the radiation intensity that excites the fluorophore. The inner-effect can be corrected by carefully selecting the concentration of the ligand, so that the absorbance of the ligand at the excitation and emission wavelength is below 0.1 (van de Weert 2010).

Collisional or dynamic quenching results from collisions involving both the fluorophore and the quencher during the lifetime of the excited state, while static quenching refers to ground-state fluorophore–quencher complex formation (Zhao et al. 2006). In the case of collisional quenching, the quencher diffuses to the fluorophore during the lifetime of the excited state and, upon contact, the fluorophore returns to the ground state without emission of a photon (Lakowicz 2006). Important parameters such as the association constant and binding number can be computed using various equations such as the well-known Stern–Volmer equation (in the case of non-fluorescent complex formation) or its modified version derived for a dynamic quenching process, in which there is a constant contribution of fluorescence of the non-quenchable fraction. These equations and variants are well described elsewhere (Zhao et al. 2006; Lakowicz 2006; van de Weert 2010).

Variants of fluorescence spectroscopy include synchronous fluorescence and fluorescence resonance energy transfer (FRET). Synchronous fluorescence is a scan that provides important information about the molecular environment in the vicinity of fluorophores in general, a shift in the position of the emission maximum corresponding to the changes of the polarity around the fluorophore (Guo et al. 2009). The synchronous fluorescence spectra is obtained by recording the fluorescence spectra resulting from the difference between the excitation wavelength and emission wavelength at 15 or 60-nm intervals, corresponding to the changes of the polarity around Tyr and Trp residues, respectively (Guo et al. 2009). Upon ligand binding, a stronger fluorescence quenching for a scan at $\Delta\lambda = 60$ nm (Trp) than for

$\Delta\lambda$ = 15 nm (Tyr) is indicative of the binding of the ligand closer to the Trp residues (Diarrassouba et al. 2013). This information is extremely important since it confirms the formation of a ground-state complex that can be further analyzed in the context of the FRET (Lakowicz 2006; van de Weert 2010). FRET occurs when there is overlap between the fluorescence emission spectrum of a donor fluorophore (in the excited state) with the absorption spectrum of an acceptor ligand (in the ground state). FRET can provide accurate structural information about protein–ligand binding. The transfer rate is affected by any condition that affects the distance between the donor and acceptor biomolecules, owing to FRET the name of 'spectroscopic ruler' (Lakowicz 2006). Thus, the Förster distance, that is the distance for a specific donor–acceptor pair where 50 % of the fluorescence energy of the donor (fluorophore) is transferred to the acceptor (ligand), can be computed to suggest the localization of the ligand on the protein (Lakowicz 2006; van de Weert 2010; Diarrassouba et al. 2013).

7.4.1 Fluorescence Emission of βlg

The fluorescence of *β*lg arises mainly from Trp and Tyr residues. At physiological pH, the dimeric form of *β*lg possesses four Trp residues, two on each monomer (Trp19 and 61). Trp61 is exposed to the solvent, thus explaining the almost complete quenching of its fluorescence. It has also been suggested that the location of Trp61, close to disulfide bond (Cys66–Cys160) and near the guanidine group of Arg124, both considered as strong quenchers, and the possible self-quenching of Trp61 of the other monomer in the *β*lg dimeric form, might all contribute to the reduction of its fluorescence emission (Stănciuc et al. 2012). Trp19 contributes the most (80 %) to the total intrinsic fluorescence, mainly due to its position in the hydrophobic cavity of the native conformation of the protein (Liang et al. 2008). It is thus a highly sensitive probe that is used for monitoring conformational modification in *β*lg. Typically, 280 nm is used as the excitation wavelength with Trp contributing to major fluorescence intensity and a maximum emission peak around 335 nm for *β*lg (Croguennec et al. 2004). While Tyr residues contribute to the fluorescence when exited at 280 nm, only the environment of Trp is studied at the excitation wavelength of 290 nm. The intrinsic fluorescence emission of *β*lg can typically be either quenched or enhanced and shift to a shorter wavelength (blue shift) or a longer wavelength (red shift). Enhancement of the fluorescence emission results from a reduction of intra molecular quenching of Trp residue, for instance when the protein unfolds (Stănciuc et al. 2012). Conversely, quenching occurs when the fluorescence emission of the Trp residue is hindered by the increased intra molecular interactions or upon binding of a ligand. The red shift of the peak indicates that the Trp residue moved from an apolar environment to a more polar region; when surrounded by a more hydrophobic environment, the peaks are blue shifted (Busti et al. 2002; Stănciuc et al. 2012).

7.4.2 Circular Dichroism

Circular dichroism (CD) is extensively used to monitor and understand the structural changes occurring during interactions of proteins with other biological molecules because of its high conformational sensitivity (Woody 2012). The CD signal is a radiation with elliptical polarization that results from the difference of unequal absorption between the left and right components of polarized light (Kelly et al. 2005; Creighton 2010). Proteins exhibit a CD spectrum that is conveniently divided into specific spectral regions, each of which is dominated by different types of chromophores and provides different kinds of information. The secondary structure can be determined by the far UV that ranges from about 170–240 nm and the information is provided by the amide group, which is the dominant chromophores (Diarrassouba et al. 2013). In the near UV range from 250 to 300 nm, protein CD is dominated by aromatic side chains and provides information about tertiary structure (Kelly et al. 2005; Woody 2012; Diarrassouba et al. 2013).

The protein CD spectrum that arises from the far UV is composed of secondary structural elements consisting of α-helix, β-sheets and unordered conformations or random coils. These structural elements in the peptide bonds undergo specific transitions which are well detailed elsewhere (Creighton 2010; Woody 2012). Briefly, it can be indicated that the α-helix characteristic CD spectrum exhibits two negative bands of comparable magnitude at about 222 and 208 nm, plus a stronger positive band near 190 nm. The β-sheet conformation is characterized by two negative bands near 217 and 195 nm and a positive band near 195 nm. Finally, the unordered polypeptides have a weak positive CD band at ∼217 nm and a strong negative band at ∼197 nm (Woody 2012; Nina et al. 2012). The near-UV CD spectra of proteins arise primarily from the packing of side-chain chromophores, including the three aromatic side chains (Phe, Tyr and Trp) and the disulfide group of cysteine (Woody 2012; Nina et al. 2012). The near-UV CD is greatly perturbed by conformational changes or ligands binding in ways that affect the geometry or environment of one or more aromatic side chains (Woody 2012; Diarrassouba et al. 2013). This information is important given that the biological functions of proteins rely on their structural characteristics, thus the smallest change in the structure may result in functional modifications.

7.4.3 βlg–Ligand Complexes

To date, four binding sites have been recognized on βlg, which include the central calyx formed by the β-barrel, the surface hydrophobic pocket in the groove between the α-helix and the β-barrel, the outer surface near tryptophan (Trp)19–arginine (Arg)124, close to the entrance of the β-barrel, and the monomer–monomer interface of the dimer (Liang et al. 2008; Yang et al. 2008; Liang and Subirade 2012). There is a wealth of literature on βlg-based complexes with ligands of

biological importance (Kontopidis et al. 2002; Creamer et al. 2011; Sawyer 2013). βlg can bind ligands of various natures, including metal ions (Divsalar et al. 2012), fatty acids (Kontopidis et al. 2002), vitamins (Yang et al. 2008; Diarrassouba et al. 2013), pharmaceuticals (Agudelo et al. 2012), flavor compounds (Tromelin and Guichard 2006) and polyphenols (Liang et al. 2008; Riihimäki et al. 2008; von Staszewski et al. 2012). This list is far from exhaustive, and research is ongoing for relevant βlg–ligand complexes.

Complexation with βlg is believed to improve the biological properties and the stability to environmental factors of both the ligand and the protein. The pepsin and acid resistance confers to βlg its functional role as carrier of small hydrophobic ligands which are thus protected during transit in the stomach. βlg then releases its cargo charge at higher pH value in the intestine upon proteolitic activity of chymotrypsin, trypsin and minor proteases present in pancreatin (Ragona et al. 2000; Keller 2013). Consequently, a function of βlg might consist in facilitating the digestion of milk fat in neonates (Ragona et al. 2000). It is important to notice that the closed conformation of the EF loop at acidic pH confirms the physiological role of βlg as a transporter of small bioactives, since bound ligands might be protected in the acidic stomach and later be released within the basic small intestine when the EF loop is in the 'open' conformation (Sakurai and Goto 2006; Creamer et al. 2011).

Evidence suggests that binding of ligands to βlg is beneficial for both the protein and bound bioactive molecule. Recently, it was proved that the resistance of βlg to proteolytic activity in the intestines was improved upon binding to vitamin D3 (Diarrassouba et al. 2014). Binding of naturally occurring phosphatidylcholine did not influence the resistance of βlg to gastric pepsinolysis but protected the protein from subsequent degradation under duodenal conditions (Mandalari et al. 2009). It has also been established that complexation with tea polyphenols extracts resulted in an increase in β-sheet and α-helix leading to an alteration of the protein's conformation, which consequently stabilized the structure of βlg (Kanakis et al. 2011). This finding was recently confirmed by the protection of the secondary structure of βlg upon binding to coffee, cocoa and tea polyphenols (Stojadinovic et al. 2013). Substantial research indicates that upon binding to βlg, hydrophobic ligands are better protected against oxidative degradation and the solubility of some of them is enhanced, which might improve the biological properties of the bioactives (Liang et al. 2008; Liang et al. 2011; Diarrassouba et al. 2013). The photostability of folic acid was improved upon binding to the surface of βlg, in the groove between the α-helix and the β-barrel (Liang and Subirade 2010). Riboflavin is a natural occurring photosensitizer that interacts with proteins located in the cell membrane and induces damage to biological systems, including tumor tissues. The binding of riboflavin to βlg leading to formation of the βlg/RF complex exhibited important antiproliferative activity, which was explained by the generation of reactive radical and oxygen species as the result of the interaction between RF and βlg (Diarrassouba et al. 2013). Additionally, it has been clearly established that βlg might have a functional advantage in the transport of vitamin D3, since supplementing milk with vitamin D3 effectively enhances its uptake (Yang et al. 2009).

The βlg–vitamin D3 complex represents an excellent model that confirms the existence of multiple binding sites on the protein. In fact, vitamin D3, α-tocopherol, as well as phosphatidylcholine, each bind to two different sites on βlg: (*i*) the surface hydrophobic pocket in the groove between the α-helix and (*ii*) the β-barrel, and central calyx (Yang et al. 2008; Mandalari et al. 2009; Liang and Subirade 2012). The binding of hydrophobic ligands to the surface site via hydrophobic interactions might provide an additional protection to βlg against proteases due to steric encumbrance (Mandalari et al. 2009). Furthermore, the existence of a secondary surface site might be beneficial to the carrier function of βlg since the disruption of the central calyx upon heat treatment can trigger the release of the bioactive bound inside the cavity (Yang et al. 2008; Liang and Subirade 2012). In fact, βlg undergoes irreversible denaturation with concomitant loss of its tertiary structure above 80 °C, which consequently disallows the binding of the ligands to the central calyx (Sawyer 2013). The binding to the central cavity of βlg can also be hampered by pH-induced transitions and particularly by the Tanford transition. At lower pH values, the EF loop—that is, the gate of the calyx—is in the 'closed conformation', thus preventing binding of the bioactives, which can have access again to the central cavity by raising the pH value (Sakurai et al. 2009; Creamer et al. 2011). Therefore, βlg can be considered as a versatile nano-sized carrier system that can conveniently transport and protect bioactive ligands, whose release can be modulated by controlling environmental factors such as the temperature and pH (Ragona et al. 2003; Liang and Subirade 2012). This property is reinforced by the surface properties of βlg to exhibit charged and neutral amino acid groups upon pH manipulation around its p*I* (5.3). At pH values close to the p*I*, βlg bears zero charge while at pH above and below the p*I*, it is negatively and positively charged, respectively. These physicochemical characteristics confer flexibility in establishing intermolecular interactions (with other βlg molecules) as well as interactions with a wide range of biopolymers. This advantageous property is exploited to fabricate βlg–biopolymer self-assembled structures used in controlled delivery technology.

7.5 The Auto-association of βlg

βlg can self-assemble to form aggregates, depending on protein concentration, ionic forces, pH and temperature of the solution. The mechanisms of βlg self-aggregation have been extensively reviewed (Meredith 2006; Nicolai et al. 2011; Foegeding and Davis 2011; Nicolai and Durand 2013). Thermal gelation of βlg has served in the development of environment-sensitive hydrogels with specific microstructural properties and desired bioactives release profiles (Chen et al. 2006; Gunasekaran et al. 2007). The advantage of thermally induced gels is their capacity to trap bioactive molecules within the gel matrix, stabilize food texture, and to swell in water and hold it in a well-maintained network structure. These characteristics confer protection from hostile environments to bioactives which can thus be released upon environmental triggers such as pH (acidic in the stomach and neutral

in the intestine), temperature and digestive enzymes (Chen et al. 2006). However, heat-sensitive bioactives cannot be encapsulated in thermal gels. This drawback can be bypassed by using cold-set gels, formed by pre-denaturing the proteins using heat treatment and subsequently using salt to induce gelation. Particulate or fine stranded gels sets can then be obtained depending on the ionic force and pH (Remondetto and Subirade 2003). The use of cold-induced gelation of *β*lg for oral delivery of nutraceuticals has been the focus of intense research (Remondetto and Subirade 2003; Remondetto et al. 2004; Sok Line et al. 2005; Chen et al. 2006; Livney 2010). The preheating of *β*lg exposes functional groups to what? which can be used to create interactions with bioactives and unfolded polypeptide chains as well as to confine the size of the delivery matrix into nano- and microparticulate systems.

The size of the biopolymer-based delivery devices can be controlled via two main processes categorized as 'top down' and 'bottom up' approaches (Augustin and Sanguansri 2009). The 'top down' approach consists of breaking up bulk materials into reduced sized matrices, whereas the 'bottom up' approach allows structures to be built from molecules capable of self-assembly (Chen et al. 2006; Augustin and Sanguansri 2009; Verma et al. 2009; Salazar et al. 2012). Food proteins, and particularly *β*lg, have great potential for self-assembly due in part to their polyelectrolytic character, which enables the possibility of conveniently manipulating their surface charge and establishing non-covalent interactions (hydrogen bonding, electrostatic and van der Waals forces). In addition, the presence of thiol groups permits covalent interactions, and lastly, hydrophobic amino acids allow the establishment of hydrophobic interactions upon unfolding of the structure (Singh 2011). The promotion of such interactions between the polypeptides of *β*lg can trigger spontaneous auto-structuration of the protein to form site-specific delivery scaffolds of controlled size.

7.6 *β*lg-Based Delivery Systems: From Molecule to Particles

Generally, nanoparticles refer to functional materials at a length scale of less than 100 nm, although a larger definition includes particles of size inferior to 1 μm (Vo-Dinh 2005; Chen et al. 2006; Sozer and Kokini 2009; Ezhilarasi et al. 2013). Beneficial features of nanoparticles include target and site-specific delivery, ability to penetrate cells and circulating systems, bioactives entrapment and dispersion throughout the dense polymeric network (Gunasekaran et al. 2007). The dense matrix formed by entanglement of polypeptide chains is believed to provide a reinforced resistance to proteolytic attack in simulated gastrointestinal conditions. As such, *β*lg nanoparticles of sub-100-nm size exhibit an improved resistance to digestive proteases at neutral and acidic pH (Gunasekaran et al. 2007). The size of the *β*lg nanoparticles can be reduced to about 60 nm upon preheating of the protein

solution. The use of crosslinking agents such as glutaraldehyde can significantly improve the density of the nanoparticle matrix, which in turn impedes the penetration of the proteolytic enzymes into the platform and subsequently retards the degradation of the protein matrix (Ko and Gunasekaran 2006). Degradation of protein-based delivery platforms by intestinal proteases is a major issue in oral delivery of bioactives, particularly for βlg-based systems. The Tanford transition at intestinal pH provides access for the enzymes trypsin and chymotrypsin to the target amino acid groups, consequently degrading the protein (Sakurai and Goto 2007; Sakurai et al. 2009; Keller 2013). Therefore, the development of βlg-based controlled-release formulations which can efficiently retard the intestinal degradation of are highly advantageous in numerous aspects: (i) increased residence time in the intestines resulting in enhanced adsorption; (ii) improved intimacy of contact with the epithelial membrane and/or at the absorption site; (iii) enhanced bioavailability (Lafitte 2008). The carrier system can be tailored to improve its attachment to the epithelial membrane at the target site, also referred to as mucoadhesion, which is important for enhanced uptake and bioavailability of biomolecules.

Mucoadhesion is an important characteristic that motivated research into βlg-based formulations with improved adhesion to the mucus layer in the intestines (Chen and Subirade 2005; Jones et al. 2009; Zimet and Livney 2009; Livney 2010;). Indeed, the mucus layer represents the first membrane barrier that covers the gastrointestinal (GI) tract, and consequently obstructs direct adhesion to the epithelial cells and hinders the transport of bioactives (Lafitte 2008). The mucus layer is a viscoelastic protective lining of the epithelium, composed mainly of water (∼95 %) and up to 5 % mucins. Mucins are glycoproteins of high molecular weight consisting of a peptide backbone with a significant number of carbohydrate side chains attached to the peptide backbone. Whereas the protein core and formation of the disulfides bridges between the peptide backbones are responsible for the hydrophobic and viscoelastic character of the mucins, respectively, the carboxylic side chains confer a strong negative charge to the mucus layer (Lafitte 2008). Recently, cationic βlg nanoparticles between 75 and 94 nm in size were developed as a bioavailability enhancer for poorly absorbed bioactives (Teng et al. 2013). The cationic βlg nanoparticles were formed by substituting 11 amino acid residues with ethylenediamine, which resulted in positive surface charge and significantly increased surface hydrophobicity. The positively charged βlg nanoparticles improved the mucoadhesion, and were proposed as bioavailability enhancers of nutraceuticals and pharmaceuticals (Teng et al. 2013).

A number of pH-sensitive, biocompatible and biodegradable polysaccharides have been used as mucoadhesive agents, and studies have shown that an increase in charge density enhances the adhesion (George and Abraham 2006; Yu et al. 2009). Interestingly, evidence also indicates that both anionic (e.g. alginate) and cationic (e.g. chitosan) can quite strongly interact with mucins. This can probably be explained by the presence of both charge groups on the backbone and side chains with different pKa, which changes the global charge of the mucin molecules when the pH varies between pH 1 and 7. Thus, while the mucins are fully negatively

charged in the intestines, they carry neutral to weak charge in the stomach, the pKa (2.6) of sialic acid (at the end of the carboxylic groups) being used as a cut-off point (Lafitte 2008). Nano-sized delivery platforms have been developed involving electrostatic complexes and coacervates formation between βlg and polysaccharides. These platforms result from the electrostatic interactions between oppositely charged molecules under particular pH and ionic force conditions (Jones et al. 2009; Chanasattru et al. 2009; Jones et al. 2010a, b; Schmitt and Turgeon 2011). Nanoparticles, with the core constituted of chitosan and shell formed by βlg were developed as nutraceutical carriers (Chen and Subirade 2005). When the native βlg was used to form the shell, the resistance to acid and pepsin degradation property of the protein was preserved.

Heat denatured βlg was used to form electrostatic complexes with pectin, an anionic polysaccharide that is only degraded in the colon by the pectinases and resists proteases and amylase in the upper GI tract (Yu et al. 2009; Jones and McClements 2010). The resulting nanoparticle can be suggested for colon delivery purposes for a wide range of bioactives including pharmaceuticals and nutraceuticals. Vitamin D2, docosahexaenoic acid and major catechin in green tea (–(–)–epigallocatechin-3-gallate) were successfully entrapped in nanoparticles of size varying from 50 to about 100 nm prepared upon promotion of electrostatic interactions between βlg and pectin (Zimet and Livney 2009; Ron et al. 2010; Shpigelman et al. 2012). The authors suggested that these nanovehicles could serve as carriers for hydrophobic nutraceuticals in non-fat foods and clear beverages. The encapsulated bioactives benefitted from significant protection against oxidative degradation, probably due to the mild antioxidant activity of βlg conferred by the free thiol group (Liu et al. 2007; Ron et al. 2010). In addition, the physical entrapment and reduced mobility of light- or oxygen-sensitive bioactives within the protein matrix might provide additional resistance to oxidizing agents such as oxygen or free radicals by restricting their access to the encapsulated bioactive molecule (Ron et al. 2010). The ability of βlg to absorb UV light can also contribute to improving the light stability of the entrapped bioactives that absorb at a proximate wavelength range (Semo et al. 2007). Nanoparticles, particularly those 100 nm or less in size, have the functional advantage of diffusing readily though both the epithelial and lymphatic tissue at target sites where the entrapped bioactive is taken up with high efficiency (Chen et al. 2006; Acosta 2008). However, the release profile of the bioactive can also be effectively modulated by larger submicron particles, which release entrapped bioactive molecules more slowly and over longer periods at the mucosal lining (Chen et al. 2006; Acosta 2008).

7.7 βlg-Based Microparticles for Oral Delivery

Microparticles are spherical shaped scaffolds, less than 1000 μm in size, isolating a variety of sensitive bioactive substances from the surrounding environment by a membrane coating (Nesterenko et al. 2013). Biopolymers and bioactive ingredients

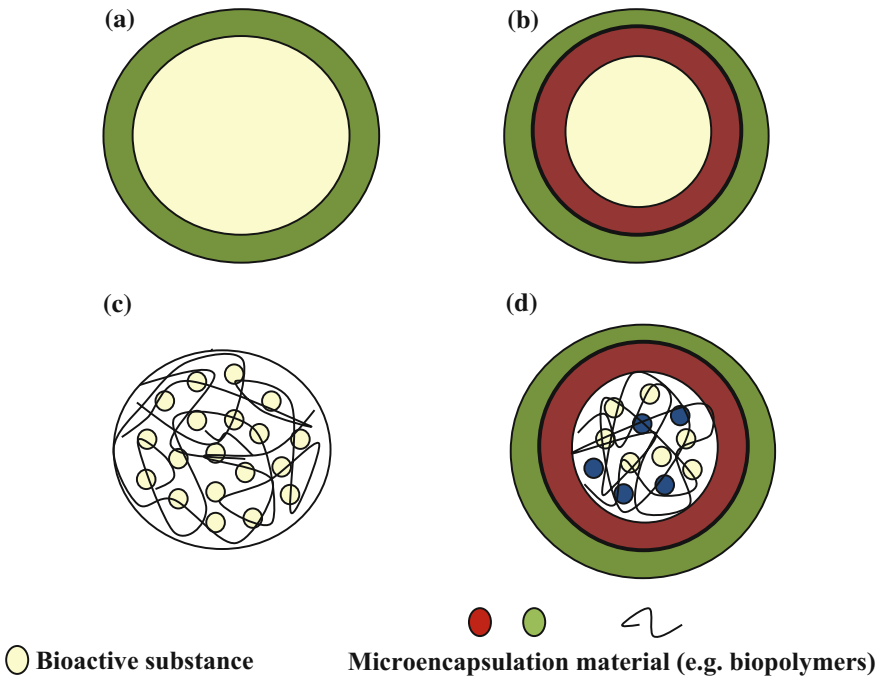

Fig. 7.2 Different structures of microparticles obtained by microencapsulation: **a** microcapsule, **b** multilayer microcapsule, **c** microsphere and **d** multishell and multicore microsphere [adapted from Nesterenko et al. (2013)]

can be combined to form different structures of microparticles including multishell, multilayer microsphere or microcapsule platforms as described in Fig. 7.2.

In the food industry, microparticles have been used for many purposes, including masking unpleasant taste and flavor, converting liquids to solids, and protecting sensitive ingredients from harsh external media including light, moisture, pH and oxidation—this list is far from exhaustive (Chen et al. 2006; Nesterenko et al. 2013). Currently, there is huge number of reports on the use of microparticles as delivery vehicles for nutraceuticals and pharmaceuticals. The use of non-toxic food-grade materials is of particular interest for oral delivery, and a vast volume of research on βlg-based microparticulate systems exists in the literature. Furthermore, βlg belongs to the lipocalin family, which explains its recognition and internalization by the human lipocalin-interacting membrane receptor that is expressed in the intestine (Sawyer 2013). Therefore, its uptake by intestinal cells is advantageous in the formulation of oral delivery platforms that entrap and release bioactives

within the intestines. Microparticles can be formulated to prolong the residence time of bioactives at the target site and improve their bioavailability as a result of enhanced mucoadhesion. Coacervates of βlg with naturally occurring polysaccharides with mucoadhesive properties, such as alginate, pectin, gum arabic and acacia gum, have been used for the encapsulation of probiotics and vitamins (Schmitt et al. 2000; Chen and Subirade 2005; Chen et al. 2006; Turgeon and Laneuville 2009; Schmitt et al. 2009; Hebrard et al. 2010).

Despite important potential applications in delivery technology, the utilization of coacervates is restricted to a narrow window of pH and ionic strength, a range beyond which the microparticles are unstable and thus can lead to premature release of the bioactive cargo charge (Turgeon and Laneuville 2009). Solutions to this issue include formation of Maillard type conjugates consisting of non-enzymatic browning involving the terminal or a side-chain amine group of βlg and the reducing end of a sugar such as dextran (Wooster and Augustin 2006; Caillard et al. 2010). Crosslinking agents including glutaraldehyde or formaldehyde have been used to stabilize coacervates; however, they are toxic and are not permitted for food applications.

Biocompatible alternatives such as rennet, genipin, glyceraldehyde or transglutaminases are all capable of stabilizing coacervates by covalently bridging protein matrices, but with a yield well below that of the toxic ones (Turgeon and Laneuville 2009; Livney 2010). However, it is important to ensure that crosslinking does not impede the bioaccessibility and release of the active molecule entrapped within the carrier matrix, which then forms a barrier against diffusion and inward access of digestive enzymes (Livney 2010). Maillard conjugates, the use of crosslinking agents for improved microparticles stability, and emulsion- and lipid-based particulate systems are beyond the scope of the present review. The formulation of bioactive-loaded carriers with controlled size and release profiles require a full understanding of the behavior of the delivery platform in food systems as well as in the GI tract at the site of absorption. The physicochemical characteristics, including surface properties such as size, surface charge, and interior and surface morphology, are important parameters to consider for optimal bio-interaction, uptake and bioavailability of the bioactive substance.

7.8 Characterization of βlg-Based Nano- and Microparticles

Most βlg-based nano- and microparticulate carriers with a dense matrix result from electrostatic complexes formed between soluble biopolymers. Typically, the self-assembly is promoted upon mixing of clear solutions under controlled pH, temperature and ionic forces conditions. This in turn leads to a modification of the light scattering (turbidity) of the resulting solution, which usually increases rapidly as a consequence of the formation of large aggregates or co-precipitates (Mahler

et al. 2009). The theory behind turbidity measurement is Rayleigh's theory, which indicates that the scattering of light is brought about by particles which are smaller in diameter than the wavelength of the light itself, the upper limit considered to be about one-tenth of the wavelength (Mahler et al. 2009; Moore and Cerasoli 2010). The increase in turbidity is normally associated with an increase in the size of the particles and is commonly measured by UV-vis spectroscopy as optical density in the 340–360-nm range and 550–600 nm (Zimet and Livney 2009; Mahler et al. 2009; Ron et al. 2010). The optical properties of a solution are a function of the particles present to scatter and absorb light (Mahler et al. 2009). However, turbidity data should be interpreted carefully, since various environmental factors including temperature, pH (near the p*I*) and protein concentration can influence the size of βlg–biopolymer self-assembled structures. The size determination is also based on the optical properties of a solution, which are dependent on the ability of the particles dispersed in the solution to scatter and absorb light. Particle size (1–10 μm) and size distribution are frequently determined using laser light scattering, which encompasses static and dynamic light scattering. Detailed information on these optical techniques has been well described in the literature (Murphy and Lee 2006; Mahler et al. 2009; Moore and Cerasoli 2010).

The surface charge of a particle is estimated using the zeta potential that is an indication of the particle stability measured by electrophoretic light scattering (Moore and Cerasoli 2010). Values of zeta potential above +30 mV and below −30 mV are indicative of a stable colloidal system. Instability arises from pH, which affects the surface charge and repulsion/attraction forces between particles, and the ionic strength, which can modify the particle's charge density (Moore and Cerasoli 2010). The interior and surface aspects of particulate systems can be characterized using transmission (TEM) and scanning electron microscopy (SEM), respectively (Chen and Subirade 2005; Chen et al. 2006; Murphy and Lee 2006). Larger-sized particles or visible particles can be observed using optical microscopes equipped with epifluorescence and optical fluorescent filters for improved discrimination between distinct biopolymers, which are stained beforehand (Chen and Subirade 2006; Hebrard et al. 2013). Finally, the encapsulated bioactive can be extracted from the carrier scaffold and its concentration quantitatively determined (encapsulation efficiency) using techniques such as UV-vis spectroscopy at specific wavelengths or high-pressure liquid chromatography (HPLC) (Zimet and Livney 2009; Ron et al. 2010).

The intestinal uptake and bioavailability of a bioactive substance developed for oral administration are commonly evaluated by performing permeability tests using cell cultures or in vivo experiments using animal or human models. The behavior of oral delivery systems in the GI tract and the in vitro bioavailability of bioactives can be monitored using in vitro digestion methods or dissolution tests, following the US Pharmacopeia/Food and Drug Administration (USP/FDA) guidelines (Acosta 2008). Briefly, digestion at 37 °C in an acidic solution (pH 1.2) in the presence of pepsin represents gastric conditions, while digestion at pH 6.8 in the presence of pancreatin simulates intestinal conditions (Chen and Subirade 2006; Acosta 2008; Hebrard et al. 2013). More specifically, the fate of the βlg-based carrier systems in

the stomach and intestines corresponds to the behavior of the protein in different compartments in the GI tract. Native βlg is resistant to acid and pepsin in the stomach, but is degraded at higher pH and by the pancreatin in the intestines (Sakurai et al. 2009; Creamer et al. 2011; Sawyer 2013). Shell/core chitosan/βlg microparticles with the shell formed by native βlg resist under gastric conditions but are degraded in the intestines by enzymes. However, microparticle shells made of denatured βlg have been shown to be rapidly degraded under both gastric and intestinal conditions (Chen and Subirade 2005).

Cell cultures with adenocarcinoma cell lines derived from human colon epithelia (Caco-2 cells) are among the most frequently used means for in vitro permeability tests. The Caco-2 cells have biophysical properties similar to those of the epithelial cells of the intestines and, as such, mimic well the absorption of the bioactives through the intestinal membrane (Acosta 2008). Therefore, Caco-2 cells can be used to evaluate the efficiency of controlled-release formulations (Remondetto et al. 2004; Jiang and Liu 2010; Sun et al. 2012). Better information on the uptake and bioavailability of a bioactive substance can be generated using in vivo experiments with rats, mice or humans. In food systems, the concept of bioavailability is usually erroneously restricted to the biopharmaceutical phase, which includes the release of the bioactive upon physical erosion or chemical degradation of the carrier system, but affords little attention to the biopharmaceutical (distribution, metabolism and elimination) and biological responses of the encapsulated bioactive (Faulks and Southon 2008). Caution should be exercised in interpreting bioavailability data resulting from in vivo experiments. Optimal bioavailability and biological response result necessarily from an accurate understanding of the relationship between the structure of the carrier platform, biophysical characteristics of both the bioactive and encapsulating material, and a good knowledge of physiology of the intended target site.

7.9 Future Trends

The development of protein-based delivery systems is attracting increasing interest, due in part to their GRAS status and the possibility for entrapping bioactives with different physicochemical properties is infinite. In particular, βlg naturally interacts with small ligands as well as food macromolecules to form complexes whose size and surface properties can be controlled. Recent reports indicate that βlg and food proteins including lysozyme, such as egg white proteins, can form electrostatic complexes at pH values between the p*I* of the two biopolymers (Desfougeres et al. 2010). Evidence strongly suggests that the self-assembly of two proteins into delivery scaffolds will constitute a significant research field in the near future, due mostly to the nutritional advantage, generation of bioactive peptides and simplicity of the process, which together offer a variety of applications including bioactive encapsulation and delivery, and protein co-precipitation and isolation. A recent study reported that βlg–lysozyme self-assembled spontaneously to form

microspheres capable of entrapping vitamin D3 at high encapsulation efficiency (Diarrassouba et al. 2014). The microspheres improved the solubility, UV light stability and shelf life in cold storage, and concomitantly enhanced the intestinal uptake and bioavailability of vitamin D3 (Diarrassouba et al. 2014).

Most importantly, protein aggregates and deposition in tissue (known as amyloid fibrils or plaques) have been directly linked with the impairment of cellular functions and the onset of degenerative neurological diseases such as Alzheimer's disease and spongiform encephalopathies (Krebs et al. 2007). The characterization and understanding of food-based protein co-precipitates may providing some insight into the mechanisms leading to the formation of these 'aberrant protein interactions' and resulting severe health conditions (Vabulas and Hartl 2011).

Particulate systems based on the auto-aggregation of βlg can be prepared by conveniently modulating the protein concentration, pH, ionic forces and temperature (Nicolai et al. 2011; Nicolai and Durand 2013). Self-aggregation of denatured βlg was induced upon the addition of calcium to form a particulate system which encapsulated α-tocopherol (Somchue et al. 2009). An alginate coating conferred reinforced protection to the βlg-based particles in the stomach and retarded the release of the vitamin in the intestines. The auto-aggregation of native βlg can be promoted by mild acidification using D-gluconic acid δ-lactone, which is advantageous for heat-sensitive bioactive encapsulation. This βlg-based platform exhibits a dense polypeptide network, in which bioactives will ultimately be bioaccessible by digestive enzymes, offering significant protective barriers against damaging environmental factors including light and oxidative species. Recently, a βlg-based platform was prepared by inducing the auto-aggregation of protein near its p*I* (Diarrassouba et al. 2014), and vitamin D3 was successfully entrapped within the matrix of platform. As a result, the solubility, UV light stability and long term storage in the cold were significantly improved.

Animal proteins have attracted much attention, mainly due to their excellent techno-functional properties, biocompatibility, biodegradability and GRAS (generally recognized as safe) status (Livney 2010; Elzoghby et al. 2012). Animal proteins collagen and gelatin, a hydrolysis product of collagen, have been widely used for oral drug delivery (Elzoghby et al. 2012). However, they have weak mechanical properties, making the use of harmful crosslinking agents such as glutaraldehyde necessary to enhance the stability of collagen and gelatin–based oral delivery systems. Although other, less toxic crosslinking agents exist, they are not readily available (Liang et al. 2003; Sehgal and Srinivasan 2009). Other animal proteins, such as silk proteins produced by larva silkworms or small insects such as spiders, and elastin or recombinant elastin, have all been used to prepare micro- or nanoparticulate oral delivery systems, which are well summarized by Elzoghby et al. (2012). However, these proteins are expensive, and producing industrial amounts may not be cost-effective. Furthermore, the implication of animal proteins in the spreading of neurodegenerative diseases such as bovine spongiform encephalitis (mad cow disease) and potential risks for allergic reactions have prompted a search for alternatives from natural sources such as vegetable proteins (Elzoghby et al. 2012).

Plant proteins are biodegradable and from abundant and renewable sources, with interesting techno-functional and physicochemical properties that are currently under intensive scrutiny for nano- and microencapsulation of bioactives (Elzoghby et al. 2012; Nesterenko et al. 2013). Protein from soy beans, peas, corn, wheat, barley, rice, oats and sunflowers have all been investigated for the development of encapsulation matrices (Chen and Subirade 2009; Chen et al. 2010; Wang et al. 2011a, b; Elzoghby et al. 2012; Nesterenko et al. 2013). For instance, zein from corn kernels, gliadin from wheat gluten and soy protein have been used for microsphere and nanoparticle formulation (Chen and Subirade 2009; Chen et al. 2010; Luo et al. 2012). However, they are highly hydrophobic, which restricts their carrier ability and they are soluble mostly in organic solvents, which may not be compatible with fragile bioactive molecules, in addition to being sensitive to the gastric environment (Elzoghby et al. 2012). Despite their reduced production cost compared to animal proteins, plant proteins are highly hydrophobic which require the use of potentially toxic organic solvents. However, this hydrophobic character is useful for avoiding the use of crosslinking agents, which is beneficial for oral delivery of bioactives (Elzoghby et al. 2012).

7.10 Sources of Further Information

- Milk proteins: Handbook of hydrocolloid 2009 (O'Regan et al. 2009; Boland 2011).
- Whey processing, functionality and health benefits. John Wiley & Sons (Onwulata and Huth 2008).
- Whey protein fractionation (Bonnaillie and Tomasula 2009).
- Detailed study on βlg structure and folding (Sakurai et al. 2009).
- Spectroscopic methods: The principle of fluorescence spectroscopy (Lakowicz 2006) and Circular Dichroism (Creighton 2010; Nina et al. 2012).
- Delivery and Controlled Release of Bioactives in Foods and Nutraceuticals (Garti 2008).

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