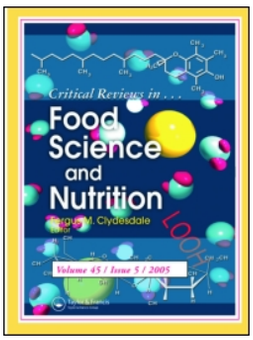

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Design of whey protein nanostructures for incorporation and release of nutraceutical compounds in food

Oscar L. Ramos[a,b], Ricardo N. Pereira[a], Artur Martins[a], Rui Rodrigues[a], Clara Fuciños[a,c], José A. Teixeira[a], Lorenzo Pastrana[c], F. Xavier Malcata[b,d], and António A. Vicente[a]

[a]CEB—Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga, Portugal; [b]LEPABE—Laboratory of Engineering of Processes, Environment, Biotechnology and Energy, University of Porto, Rua Dr. Roberto Frias, Porto, Portugal; [c]Biotechnology Group, Department of Analytical Chemistry and Food Science, University of Vigo, Ourense, Spain; [d]Department of Chemical Engineering, Rua Dr. Roberto Frias, Porto, Portugal

ABSTRACT

Whey proteins are widely used as nutritional and functional ingredients in formulated foods because they are relatively inexpensive, generally recognized as safe (GRAS) ingredient, and possess important biological, physical, and chemical functionalities. Denaturation and aggregation behavior of these proteins is of particular relevance toward manufacture of novel nanostructures with a number of potential uses. When these processes are properly engineered and controlled, whey proteins may be formed into nanohydrogels, nanofibrils, or nanotubes and be used as carrier of bioactive compounds. This review intends to discuss the latest understandings of nanoscale phenomena of whey protein denaturation and aggregation that may contribute for the design of protein nanostructures. Whey protein aggregation and gelation pathways under different processing and environmental conditions such as microwave heating, high voltage, and moderate electrical fields, high pressure, temperature, pH, and ionic strength were critically assessed. Moreover, several potential applications of nanohydrogels, nanofibrils, and nanotubes for controlled release of nutraceutical compounds (e.g. probiotics, vitamins, antioxidants, and peptides) were also included. Controlling the size of protein networks at nanoscale through application of different processing and environmental conditions can open perspectives for development of nanostructures with new or improved functionalities for incorporation and release of nutraceuticals in food matrices.

KEYWORDS

Whey proteins; aggregation; gelation; Nanostructures; Nutraceuticals; Encapsulation

1. Introduction

Milk proteins, and whey proteins in particular are widely used as ingredients in formulated foods because they are valuable by-products, relative inexpensive, generally recognized as safe (GRAS), and have high nutritional value—due to their high content of essential amino acids especially sulfur-containing ones (de Wit, 1998; Ramos et al., 2012a). Moreover, these proteins have important biological (e.g. digestibility, antimicrobial, antiviral and anticarcinogenic activity, amino acid pattern and immune system modulation), physical and chemical (e.g. water absorption, gelation, foaming and emulsifying) functionalities essential in food applications (Dickinson, 2003; Gunasekaran et al., 2007; Madureira et al., 2007; Dissanayake and Vasiljevic, 2009; Sarkar et al., 2009). Among them, gelation is particularly interesting; it involves different physical and chemical transformations, depending on the prevailing intrinsic and extrinsic factors related to charge density and hydrophilic–hydrophobic balance (Ramos et al., 2012b). Gels of diverse mechanical and microstructural properties can be prepared from whey protein solutions by controlling the assembly of protein molecular chains, simply through adjusting a few gelation variables (e.g. concentration, temperature, pH, ionic strength and electric fields); thus offering the possibility of developing GRAS biocompatible carriers for controlled release of biologically-active substances (e.g. nutraceuticals) in a wide variety of foods (Gunasekaran et al., 2006).

One of the most recent vectors used for controlled release of nutraceutical compounds in food products are through the use of nanostructured systems (Cerqueira et al., 2014). The technology involved in the manufacture, processing, characterization and application of such systems has the ability to control the shape and size of materials at the nanometer scale (Chau et al., 2007; Bouwmeester et al., 2009). Since nanostructures are submicron and sub-cellular in size, they have versatile advantages for targeted, site-specific delivery purposes as long as they may penetrate circulating systems and reach specific sites in the body (Vinogradov et al., 2002; Cerqueira et al., 2014). The properties of materials at this scale can be very different from conventional-sized materials manufactured from the same substance. This behavior is due to the large surface area-to-volume ratio typically found in such nano-materials, but also to physical and chemical interactions between materials at the nanoscale that have a significant effect upon the overall properties of those systems (Kaya-Celiker and Mallikarjunan, 2012). This nano-scale range can change or enhance properties, such as strength, reactivity and electrical characteristics, thus providing different or new functionality to existing products—e.g. allow specific delivery and controlled release of nutraceuticals in food

CONTACT Oscar L. Ramos oscar.ramos@ceb.uminho.pt CEB—Centre of Biological Engineering, University of Minho, Campus de Gualtar, P-4710-057 Braga, Portugal.

matrices, and improve adhesion to and absorption rates through cells (Chen et al., 2006; Chaudhry et al., 2010).

The major nanostructured systems made from whey proteins (e.g. nanohydrogels, nanofibrils, and nanotubes) are unique because, in addition to their GRAS properties and gelling capability, they can be easily prepared and the size distribution effectively monitored. These protein nanostructures have also the ability to interact with a large variety of nutraceuticals via either primary amino groups or ionic and hydrophobic binding, control the release rate of nutraceuticals by swelling behavior of gel in response to environmental condition changes (e.g. pH, temperature or electric fields), protect sensitive compounds from degradation and control their bioaccessibility to digestive enzymes, and consequent bioavailability (Chen et al., 2006; Chen and Subirade, 2006; Matalanis et al., 2011; Livney, 2010; Livney, 1992). Moreover, several changes can be induced in the whey protein matrix allowing formation of complexes through interactions with other biopolymers, mostly polysaccharides, as a base for several nanosystems, allowing a synergistic combination of properties.

Nutraceuticals are a category of compounds that has received increasing attention in recent years, by both the scientific community and the market at large. Besides antioxidants, the list of nutraceutical compounds includes vitamins, prebiotics, probiotics, fatty acids and bioactive peptides—and scientific evidence supporting their therapeutic potential, and associated health benefits is steadily growing (Wildman, 2006; Cencic and Chingwaru, 2010; Chen et al., 2014). Most pathways of nutraceuticals when performing physiological functions in the human body have not yet been fully elucidated; however, it is well recognized that their addition to food products aids in preventing the risk of disease, especially chronic diseases and inflammation, so they hold a strong promise in terms of public health (Cencic and Chingwaru, 2010; Brown, 2014; Chen et al., 2014).

However, the effectiveness of these compounds in providing physiological benefits depends on their stability, during food processing, and eventual bioavailability; hence, their incorporation in nanostructured systems appears to be a suitable solution to preserve activity until the time of consumption, and deliver to the cellular target in the human organism upon ingestion (Cerqueira et al., 2014).

This review intends to bring new insights about the different stages involved in the production of different whey protein nanostructures. Classical concepts regarding the events that precede the development of whey protein networks, such as molecular interactions, denaturation, and aggregation pathways will be addressed together with the effects of using innovative and emergent processing technologies. The increasing interest from food and pharmaceutical industries in the production of whey protein nanostructures aiming a strong nutraceutical function through the incorporation of bioactive compounds will be also addressed throughout this review.

2. Whey proteins

Whey proteins are widely accepted as food elements (used as ingredient in confectionery, bakery and ice cream products, infant formula, health foods, and sports bars). A wide variety of commercially finished whey products are now available in the market including whey protein concentrates (WPC), with a protein content between 50 and 85% on a dry basis, whey protein isolate (WPI) with a protein content above 90%, and very small amounts of lactose and fat (Huffman, 1996; Ramos et al., 2013). Whey proteins are also appropriate matrices for delivery of bioactive compounds, and are accordingly a remarkable component of human diet. They have thus received considerable attention, both as potential delivery vehicles and as precursors of bioactive peptides that may form even during digestion (Zimet and Livney, 2009; Livney, 2010; Nagpal et al., 2011; Relkin and Shukat, 2012). These proteins are typically globular in nature (very susceptible to denaturation by heat), with high levels of secondary and tertiary structures in which the acidic/basic and hydrophobic/hydrophilic amino acids are distributed in a fairly balanced way (Smilowitz et al., 2005).

The major components of whey proteins are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulin (IG), and bovine serum albumin (BSA), representing 50, 20, 10, and 10% of the whey fraction, respectively. Whey contains also numerous minor proteins such as lactoferrin (LF), lactoperoxidase (LP), and proteose peptone (PP), together with other minor components (Jovanovic et al., 2007; Santos et al., 2012). The whey proteins profile, including general chemical and physiochemical properties, is shown in Table 1.

3. Development of whey protein networks

Depending on their environment and molecular architecture, whey protein molecules can assemble into a range of different structures. The kind of protein–protein interactions (chemical and physical), aggregation mechanisms and types of protein aggregates, gelation mechanism, as well as processing

Table 1. Composition of major proteins in bovine whey, relative concentration, molecular weight (Mw), isoelectric point (pI), temperature of denaturation (Td) and number of amino acid residues.

Whey protein	Concentration (g L^{-1})	Mw (kDa)	pI	Td (°C)	Number of amino acid residues
Total	7	—	—	—	—
β-Lactoglobulin	3.5	18.3	5.2	71.9	162
α-Lactalbumin	1.2	14.2	4.8	64.3	123
Immunoglobulins	0.7	150–900	5.5–6.8	—	—
Bovine serum Albumin	0.4	66.4	4.7–4.9	72.0–74.0	583
Proteose peptones	≥1	<12	3.3–3.7	—	—
Lactoferrin	0.02–0.35	80.0	8.0–8.5	63.0 and 90.0	700
Lactoperoxidase	0.01–0.03	78.5	9.8	70.0	612

Note: (–) Variable value.

techniques, determine much of the nanostructures that can be formed. The molecular approach to whey protein aggregation is important as it clarifies details of association mechanisms and of gelation process that impact strongly upon the design, development and performance of nanostructures in food materials, once affects the gel formation and strength, and thereof nanostructure's size, morphology, binding capability and functionality. These topics will be reviewed in the following subsections.

3.1. Protein–protein interactions

Certainly one of the most important characteristics of whey proteins (and particularly in the case of β-Lg and α-La) is the protein–protein interaction ability. β-Lg interaction with other milk proteins as caseins and α-La is well known (Elfagm and Wheelock, 1978; Ye et al., 2004), and allows development of tailored functional protein matrices with novel rheological and emulsifying properties (Famelart et al., 2003; Pizones et al., 2014). In spite of the versatile binding and interaction possibilities of β-Lg with other molecules, the interaction with itself as a pure protein or as the major component in WPI or WPC has been, in the last decades, one of the most reviewed subjects and the one having the most technological implications. Understanding the forces that govern protein stability is crucial to design and control the interactions of complex biological molecules, such as whey proteins. Different types of molecular interactions established between the major whey protein molecules were reviewed by several authors (Bryant and McClements, 1998; Nicolai et al., 2011; Edwards and Jameson, 2014). Electrostatic interactions, hydrogen and disulphide bonds, hydration and hydrophobic effects are intrinsic forces that determine stability of the tertiary folds of native globular proteins, as well their interfacial behavior and interaction with other molecules. However, the contribution of different types of bonds to the protein aggregation process is not yet fully understood (de la Fuente et al., 2002; Nicolai et al., 2011). It is generally accepted that a large proportion of high-molecular-mass whey protein aggregates are formed by intermolecular sulphydryl/disulphide exchange reactions, leading to formation of intermolecular disulphide covalent bonds (Sawyer, 1968; Shimada and Cheftel, 1989; Hoffmann and van Mil, 1997; Foegeding and Davis, 2011). However, non-covalent interactions such as ionic, electrostatic, van der Waals and hydrophobic are also involved in aggregation phenomena, playing an important role in the propagation step (Foegeding and Davis, 2011; Nicolai et al., 2011).

3.1.1. Noncovalent bonds

Noncovalent bonds are typically reversible and relatively weak but when existing on a larger and cooperative scale, the overall strength of interaction can be large. In particular, the contribution of noncovalent interactions becomes of increasing importance at pH values closer to the isoelectric point (pI) or under higher salt concentrations (Hoffmann and van Mil, 1997; Karshikoff, 2006d).

Electrostatic interactions are highly influenced by pH and ionic strength of surrounding aqueous solution, being responsible for the stabilization of globular proteins. These interactions can be manipulated by adjusting pH to pI and/or addition of ions (Kinsella and Whitehead, 1989; Karshikoff, 2006a). The magnitude and range of these interactions can be reduced considerably in the presence of electrolytes due of electrostatic screening induced by the counter-ions (Kitabatake et al., 2001; Karshikoff, 2006a). Attractive interactions between protein molecules increase in strength with increasing temperature due to their entropic origin (Karshikoff, 2006b). Processing by heat or pressure may induce changes in the protein structure making its peptide chain more mobile. As a consequence, unfolded molecules may interact through hydrophobic interactions or by forming hydrogen bonds, thus leading to aggregation (Nicolai et al., 2011).

Hydrogen bonds stabilize the aggregates formed, but are not usually the major driving force determining conformation and aggregation of globular proteins (Croguennec et al., 2004).

Intermolecular hydrophobic interactions are responsible for the stability and structure of β-Lg. Despite the largely hydrophilic nature on its surface, β-Lg exhibit a significant number of apolar residues that become accessible to solvent molecules during thermal treatment, for example. In fact, this kind of interactions is temperature-dependent, and also the main driving force responsible for control of protein aggregation. One of the characteristic features is their tendency to increase in gel strength as temperature is raised (De Wit, 1990; Karshikoff, 2006c; Nicolai et al., 2011).

Hydration interactions prevent protein molecules from aggregating, since stronger repulsion and longer range of interaction are promoted when hydration level is high (Edwards and Jameson, 2014).

Steric interactions are intrinsically related to possible conformations of proteins in solution, in this regard, protein molecules cannot adopt any spatial arrangements in which two or more segments occupy the same space. There is an extremely strong repulsive interaction between atoms or molecules at close separations because of the overlap of their electron clouds. This determines how closely they can pack together, besides defining the size and shape of molecules (Edwards and Jameson, 2014).

Van der Waals interactions seem to present similar magnitudes regardless of protein conformation state (folded or unfolded), but play a minor role in aggregation. However, if the protein molecule is large enough to act as a colloidal particle, then aggregation with other biopolymer molecules is likely due to strong van der Waals attraction (Bryant and McClements, 1998; Nicolai et al., 2011).

3.1.2. Covalent bonds

Interchange reactions between free sulphydryl and disulphide bonds are considered crucial for initiation of aggregation and gelation of proteins, and are mainly governed by formation of oligomers that combine into aggregates (Shimada and Cheftel, 1989; Mulvihill et al., 1991; McSwiney et al., 1994; Hoffmann and van Mil, 1997; Livney and Dalgleish, 2004). Classical theories suggest that disulphide bonds stabilize proteins by reducing entropy of the denatured state (Betz, 1993). Heating of whey proteins at denaturation temperatures induces molecular unfolding of their native structure, which leads to exposure of free sulphydryl from cysteine121 that was initially buried in the native state. Once exposed to the aqueous phase, this group is

able to form disulphide bonds with other free sulphydryl groups or by reacting through sulphydryl/disulphide interchange reactions with existing disulphide bonds (that are present in β-Lg and α-La). These intermolecular disulphide bridges are involved in aggregation processes of β-Lg and α-La (when isolated), or in co-aggregation of β-Lg with α-La (when together).

3.2. Aggregation

Whey protein aggregates serve as "building blocks" for design and development of food-grade micro- and nanonetwork structures. In the food industry, micron-size whey protein aggregates can be used to produce hydrogels with swelling behavior, which may act as texturizing agents or fat replacers. In turn, nanosize whey aggregates can improve the stability of protein foams and emulsions (Guilmineau and Kulozik, 2006). Nanostructured systems made from whey proteins have also the capability to conjugate nutrients via either primary amino groups or ionic and hydrophobic binding (Chen et al., 2006), thus offering the possibility for development of GRAS biocompatible carriers aimed at oral administration of sensitive bioactive compounds in a wide variety of foods.

In solution, proteins exist in equilibrium between two states: the native, more compact, organized and stable; and denatured, more random, disordered and reactive (Bryant and McClements, 1998). The proteins conformation and its interactions depends on the delicate balance between opposite forces that favor both states, thus the free energy between the two states is very small and very dependent of the environmental conditions. Therefore, the aggregation process in whey proteins is usually preceded by a step to favor the denatured state; without this step, protein network structures would be harder to achieve and once formed remain hardly stable in water (Pérez-Gago et al., 1999; Bodnár et al., 2007; Ramos et al., 2012a). A quantitatively kinetic model for the temperature-induced denaturation and aggregation of β-Lg, in almost neutral conditions, has been previously presented (Roefs and De Kruif, 1994). Most heat-induced changes of whey proteins have been carried out on β-Lg since it is the most abundant protein in whey, thus affecting functional properties of whey protein products. This model recognizes an initiation, a propagation and a termination step by analogy with polymer radical chemistry, in which the free sulphydryl plays the role of the radical. Initiation starts with a reversible reaction, in which native β-Lg dimer splits into monomer, followed by exposure of the free sulphydryl group. This results in the formation of active monomers giving rise to an irreversible reaction. The propagation step corresponds to the buildup of aggregates through sulphydryl/disulphide reactions (covalent bonding). In the termination step, two active intermediates react to form larger aggregates without exposing any reactive sulphydryl group.

The denaturation and aggregation behavior is classically induced by heating above the denaturation temperature of the proteins, but some other physical and chemical processes have achiever similar effects in denaturation and aggregation induction. Besides thermal effect, physical means includes pressure and electrical fields. On the other hand, chemical means includes pH changes, enzymatic actions, and use of salts and denaturation agents (e.g. urea). All this means cause changes in protein–protein and protein–environment interactions and may result in different protein structures (Totosaus et al., 2002).

3.2.1. Effects of processing on whey protein aggregation: Emerging technologies

It is well established that heat processing causes changes in the physicochemical properties of milk proteins. Despite no noticeable heat-induced effects are observed on the structure of the casein micelle fraction in the temperature range 70–100°C, heating does have a detrimental effect on the whey protein fraction of milk. Some of the most important physicochemical changes in milk proteins by heating include: whey protein denaturation and its interaction with casein micelles (Morr, 1985). Heating milk at pasteurization temperatures (>70°C) containing whey proteins, such as β-Lg, α-La, and casein micelles, results in the formation of a heterogeneous complex between these protein species (Cho et al., 2003). Moreover, the method under which milk is heated (direct/indirect or slow/rapid) appears to influence the denaturation and aggregation of whey proteins and their association with the casein micelles (Corredig and Dalgleish, 1996; Pereira et al., 2011).

The effects of thermal processing in milk and in the particular case of whey proteins, have been extensively studied either in the dairy industry as in the technological potential of whey protein structures. Although, most of the works preformed in this field focus on conventional processing technologies as heat exchanging. Emerging processing technologies are opening new perspectives in food processing due to higher process and energy efficiencies, conjugated with higher product quality. This new technologies are proving to be new and innovative ways to promote protein functionalization and open new perspectives in protein structure design.

3.2.1.1. Microwave. Microwave (MW) is an example of dielectric heating, in which an alternating electromagnetic field interacts with polar molecules, such as water and ionic species, forcing them to constantly realign themselves by reversing an electric field around the food product, thus resulting in heat generation (Pereira and Vicente, 2010). This molecular movement is extremely fast due to the high frequency of the field that can range from 300 to 3000 MHz. MW is primarily a radiation phenomenon that usually takes place in a restricted space, or in close vicinity to a waveguide applicator, where a wave is propagated and reflected (Leadley, 2008).

Possible effects of these electromagnetic fields on biological systems have been a hot discussion topic for long time. In spite of biological effects have been reported (Banik et al., 2003) the difficult dissociation of heat generation do not leave clear the nonthermal effects of this technologies. In particular, higher whey protein denaturation have been reported during MW heating when compared with conventional method, as well as having an effect in the structure and unfolding pathways of β-Lg, suggesting a synergetic or even no thermal effect (Villamiel et al., 1996; Gomaa et al., 2013). Exposure to MW radiation have also demonstrated to alter protein conformation without bulk heating, enhance of protein aggregation, and

promote specific structure formation as amyloid fibrils (de Pomerai et al., 2003).

MW heating is often reported to cause nonuniform heating, due to its poor penetration capacity which may lead to nonuniform processing. This nonuniform heating along with the complexity, high equipment costs, inability to ensure homogeneity and lack of suitable packaging materials are the major drawbacks in the MW prospecting. Thus, may result in several issues related not only with safety, but also with poor final quality and overheating (Vadivambal and Jayas, 2010).

3.2.1.2. High-voltage electric field. High Voltage Electric Fields (HVEF) is a food processing technology that can ensure the safety of the product while preserving its characteristics due to minimal detrimental impact imposed by the electric current (Mohamed and Eissa, 2012). Applications include the use of pulsed electric fields (PEF) for nonthermal sterilization (Toepfl et al., 2007) or electric fields to change protein molecules (Cramariuc et al., 2005; Zeng et al., 2008). With regard to this, PEF of high intensity (typically 20–80 kV cm^{-1}) can modify the structure/function of whey proteins in order to specific and/or desired functional properties in a similar manner to the used for controlled heat treatments. Several mechanisms may help explaining the influence of electric fields upon the molecular structure of proteins, such as: polarization of protein molecule; dissociation of noncovalently linked protein sub-units involved in quaternary structure; changes in protein conformation so that hydrophobic amino acid or sulphydryl groups become exposed; attraction of polarized structures by electrostatic forces; and hydrophobic interactions or covalent bonds forming aggregates (Castro et al., 2001).

PEF technology presents clear advantages as it has low energy requirements and the possibility of induce modification of the structure and functionality of proteins without heat side effects, as thermal degradation of liable compounds. Nevertheless, heating prevention is not always possible and the nature of the electric pulses (i.e. high voltage) turn out difficult the full control and automation of the process. In addition, the high investment cost, as well as the cost of intensive maintenance and service of PEF equipment inhibit a broad industrial exploitation of this technology (Toepfla et al., 2006).

3.2.1.3. Moderate electric fields. The Moderate Electric Fields (MEF) technology can be distinguished from other electrical heating methods by: (i) The presence of electrodes contacting the foods; (ii) The frequency applied (ranging from 50–25,000 Hz); and (iii) The unrestricted, though typically sinusoidal, waveform (Machado et al., 2010). MEF is a process characterized by application of electric fields of relatively low intensity (arbitrarily defined between 1 and 1000 V cm^{-1}) when compared with PEF, and can be used in combination with heating (ohmic heating) in the production of protein-based structured systems, such as edible nanosystems (e.g. nanohydrogels, nanofibrils, and nanotubes). MEF provide uniform and extremely rapid heating rates of liquids, which enables the application of higher temperatures without inducing coagulation or excessive denaturation of proteins (Parrott, 1992). Given the complex biochemical structure of whey proteins, the influence of MEF on their denaturation and aggregation has been recently assessed. It has indeed been demonstrated that MEF processing offers the potential to reduce whey protein denaturation at relatively high temperatures, during the early stages of heating (Pereira et al., 2011). Through application of MEF, denaturation reactions appear to be less dependent on temperature increase, thus improving thermo-dynamical stability of whey proteins. In this sense, MEF technology may play a major role on interfacial, aggregation and gelation properties of whey proteins, which are intrinsically related to protein denaturation and protein–protein interactions. Application of MEF for manufacture of WPI nanostructures has been recently reported (Pereira et al., 2010; Rodrigues et al., 2015). In agreement with these studies, MEF caused a smaller increase of whey protein's aggregate size. In particular, WPI solutions treated at 0 and 10 V cm^{-1} presented an average particle size of 86.0 $\pm$ 0.5 and 76.6 $\pm$ 0.5 nm, respectively. This study also shows that reactive free sulphydryl (responsible for initiation of aggregation pathway's) are affected by the presence of an alternating electric field, i.e. samples treated at 10 V cm^{-1} exhibited less 2.2 micromoles of sulphydryl per gram of WPI than samples treated at 0 V cm^{-1}. From the observed results it was concluded that MEF produced always smaller structural changes during denaturation and aggregation pathways of whey proteins. This peculiar denaturation and aggregation behavior of whey proteins under MEF has been attributed to conformational disturbances on tertiary protein structure due to rearrangement of hydrogen bonds, hydrophobic interactions, and ionic bonds. Further, noncovalent interactions may also be impaired by reorientation of hydrophobic clusters occurring in the protein structure during MEF application, thus affecting physical aggregation. Authors mentioned that MEF treatment may also affect ionic movement in the medium, and modify the molecular environment due to the increased number of ions and their different distributions around the protein molecules. Alternatively, the combined effects of MEF and sinusoidal frequency may promote splitting of large aggregates induced by thermal processing, thus enhancing formation of small particles. Moreover, during MEF treatment, heat is generated directly within the sample (internal volumetric heating) and hence the problems associated with heat transfer surfaces are eliminated, which may contribute to lower rates of whey protein denaturation, particularly at higher temperatures. Because of the opposite effects of these treatments (thermal and electric), has been reported that may be possible to control the size of whey protein nanostructures by simultaneously controlling temperature and intensity of the applied electric field. Currently, MEF technology is available commercially under the form of ohmic heaters adapted to a wide variety of products with the main focus on thermal pasteurization. As established technology and with proven efficient in processing and energy efficiency, as well as in inherent products quality, MEF technology needs step forward to exploit non thermal-focused applications. Difficulty with dissociation MEF with heat generation and little understanding of the influence of process parameters, such as wave type (e.g. sinusoidal and quadratic) and frequency, are some of the current limitations in MEFs application range. Overall, the use of MEF may open a new perspective for the manufacture of protein nanostructures with new functional and technological properties.

3.2.1.4. High pressure. Isostatic high pressure (HP) could as well be used for food texture engineering due to its influence on the properties of food proteins. The HP stability of individual proteins is linked to their size; oligomeric proteins dissociate at low pressures (<200 MPa), whereas unfolding of monomeric proteins is usually observed at pressures higher than 400 MPa (Mozhaev et al., 1996). The denaturation of α-La, determined by loss of solubility at pH 4.6, is observed at pressures higher than 400 MPa (>100 MPa for β-Lg) (Lopez-Fandiño et al., 1996; Tanaka and Kunugi, 1996; Huppertz et al., 2006). The more rigid molecular structure of α-La (relative to β-Lg) is the main factor for its higher barostability. The stiffness of the protein molecule is conferred by a greater number of intramolecular disulphide bonds (two more than β-Lg), and to the absence of the free sulphydryl, which can take part in sulfhydryl-oxidation interchange reactions of sulfhydryl-disulphide (Huppertz et al., 2006). HP can lead to the irreversible denaturation of proteins and production of gels glossy that retain their original flavor and color, unlike heat-induced gels (Jaeger et al., 2012). Aggregation and gelation of aqueous solutions of β-Lg (pH 7.0; 100 to 140 g kg^{-1} protein) have been induced by pressure application and release at 450 MPa (25°C, 15 min) (Dumay et al., 1998). However, these authors have observed that pressure-induced aggregation led to porous gels prone to exudation, in contrast to heat-induced gels displaying a finely stranded network with high water retention. Pressure denaturation of proteins is considered a complex phenomenon that depends on a number of factors, such as protein structure, pressure range, temperature, pH, and solvent composition (Masson 1992; Jaeger et al., 2012). In spite of proven its value in protein functionalization the high complexity of the process along with low information of the fundamental principles involved, require extensive work to HP validation as a tool in bioscience. Other issues as high costs of the equipment, high maintenance requirements and scale-up limitations also contribute to limit the technology applicability (Toepfla et al., 2006).

3.3. Gelation

Gelation of proteins usually requires a driving force to unfold the native protein, followed by an aggregation process to produce a three-dimensional network. In this sense, the driving force for gelation can be either a chemical (e.g. acid-, ionic- or enzyme-based) or physical (e.g. heat and pressure) process (Stokes, 2012). Nerveless, the acid, ionic, and enzymatic gelation often requires an initial physical pre-treatment (i.e. heating), which will help to unfold native structure of protein molecule, exposing it to further reactions. Despite these gelation methods being often used in combination, the most common used to form food gels with globular proteins is heating (Foegeding, 2006). Thermal gelation is a phenomenon that typically encompasses three stages: primary aggregation through covalent (e.g. disulfide bridges) and noncovalent bonds (e.g. hydrogen bonds and hydrophobic, van der Waals interactions); secondary aggregation with association between protein primary-aggregates; and finally formation of a three-dimensional network able to entrap water, when the amount of protein secondary aggregates exceeds a critical concentration. This continuous building process is called "gelation" and results in formation of several protein network structures at nanoscale such as nanohydrogels, nanofibrils, and nanotubes (Rubinstein and Colby, 2003). Ferry (Ferry, 1948) proposed a general model for globular protein gelation; this model describes gelation based on denaturation temperature, and a critical protein concentration and gelation time that is dependent on the rate of denaturation and aggregation.

Protein hydrogels can be produced either by physical or chemical gelation. Both forms show heterogeneous organization of independent domains, although they differ in nature of molecular associations forming the network. Physical gels are organized in heterogeneous clusters of distinct domains formed by molecular entanglements, free chain ends, and molecular "hairpin," "kinks," or "loops" held together by noncovalent bonds (Hoffman, 2002). Physical gels exhibit high water sensitivity (degrade and even disintegrate completely in water) and thermo-reversibility (melt to polymer solution when exposed to heat).

Chemical hydrogels (also called "irreversible" or "permanent" gels) are networks of polymer chains covalently linked at strategic connection sites. Most commonly, crosslinking is not spontaneous, but deliberately induced by reaction with such small molecules as aldehydes (Hoare and Kohane, 2008), radiation or UV light (Jo et al., 2005). Uneven distribution of crosslinking within the gel leads to development of some zones in which typical "reversible" features are still dominant, and other zones with permanent properties arising from the crosslinked network. Chemical hydrogels neither disintegrate nor dissolve in aqueous solutions. They rather hydrate and swell until an equilibrium state is reached, which in turn depends strictly on the extent of crosslinking.

3.3.1. Influence of environmental factors on protein gelation

The occurrence and extent of protein aggregation, and thus of gelation can be controlled by the heating processing conditions, such as time versus temperature treatment applied (in terms of level of denaturation imposed). However, together with temperature, a variety of other environmental factors can significantly impact the aggregation behavior of proteins. These include the chemical environment of aqueous solution (pH, protein concentration and ionic strength), and/or addition of electrically charged species (cold gelation) (Debeaufort et al., 1998; Pereira et al., 2010). Development and production of whey protein nanosystems can be tailored by small changes in these external factors (Mulvihill and Kinsella, 1988; Lefèvre and Subirade, 2000; Remondetto et al., 2002).

3.3.1.1. Temperature. Temperature, when increased, promotes several additional destabilizing effects upon thermodynamic stability of whey proteins. These effects include reduction of activation energy, increase of protein diffusivity and frequency of molecular collisions, and enhancement of hydrophobic interactions, which are necessary steps for physical protein aggregation. Consequently, high temperature (above protein denaturation level) is a common parameter selected for accelerating whey protein aggregation (Bryant and McClements, 1998). Generally, globular proteins such as β-Lg aggregate spontaneously and irreversibly if they are denatured at heating temperatures above 60°C (Pereira et al., 2011; Nicolai and Durand, 2013). At denaturation temperatures, whey

proteins start to unfold and, depending on the balance between attractive and repulsive interactions, they can remain as individual denaturated molecules or form fibrillar or particulate aggregates (Nicolai et al., 2011). These outcomes are extremely dependent both on the heating method (direct and indirect) and heating conditions, such as temperature, heating rate and treatment time. Temperature is the most important and widely used condition in physical gelation to produce protein nanohydrogels, as it promotes denaturation (unfolding of polypeptide chains, with concomitant exposure of initially buried hydrophobic amino acid residues) and subsequent aggregation of protein molecules into a network (Chen et al., 2006). The typical time-temperature needed for protein nanostructure preparation ranges from 10–60 min and 60–90°C, depending of the whey protein used—see Table 1.

3.3.1.2. pH. The type and distribution of surface charges on proteins is determined by pH. This parameter affects both intramolecular folding and intermolecular protein-protein interactions. Therefore, pH along with sequence hydrophobicity and propensity to form secondary structures are key parameters in determining the rate of protein aggregation, and important factors to take into account for controlling strength of physical nanohydrogels—see Fig. 1 (Bryant and McClements, 1998; Ramos et al., 2012a). The balance between pH and ionic strength on whey protein solutions has been widely investigated for the production of different types of hydrogels—transparent or turbid gels (Ramos et al., 2012b). The so-called fine-stranded gel is composed of finely stranded nanometer-thick networks, exhibiting a transparent or translucent appearance and a rubbery texture. It is formed under conditions where intermolecular electrostatic repulsion is dominant, which occur at low ionic strength and at pH values far from the protein pI (Nicolai et al., 2011; Ramos et al., 2012a). Intermolecular repulsion can be screened by shifting pH towards pI (pH range 4 to 6) or by increasing ionic strength. At these conditions, aggregation is accelerated by heat, thus leading to formation of turbid or white opaque gel composed by micrometer-sized particulate random aggregates—see Fig. 1 (Chen, 1995; Gounga et al., 2007; Sanghoon and Sundaram, 2009).

3.3.1.3. Protein concentration. The effect of protein concentration on aggregation has been evaluated extensively (Bryant and McClements, 1998). Regarding whey proteins, increasing protein concentration often produces: (1) increased aggregation due to increased chance of protein–protein interactions; and (2) precipitation due to solubility limit. Concentration plays an important effect in protein aggregation, particularly when denaturation is induced by heat; in this case, the tendency of a protein to aggregate is higher when its concentration is high (Wehbi et al., 2005). Equally, when a salt is added to a heat-denatured protein solution, the concentration of protein has a major influence on the rheological properties of the solutions; at low protein concentrations the heat-denatured protein will tend to form a viscous solution—yet above the critical protein concentration, a gel is obtained (cold gelation) (Remondetto et al., 2002; Ramos et al., 2012b).

3.3.1.4. Ionic strength. Ionic strength is another condition that strongly affects protein aggregation. Both positive and negative ions can potentially bind or interact electrostatically with

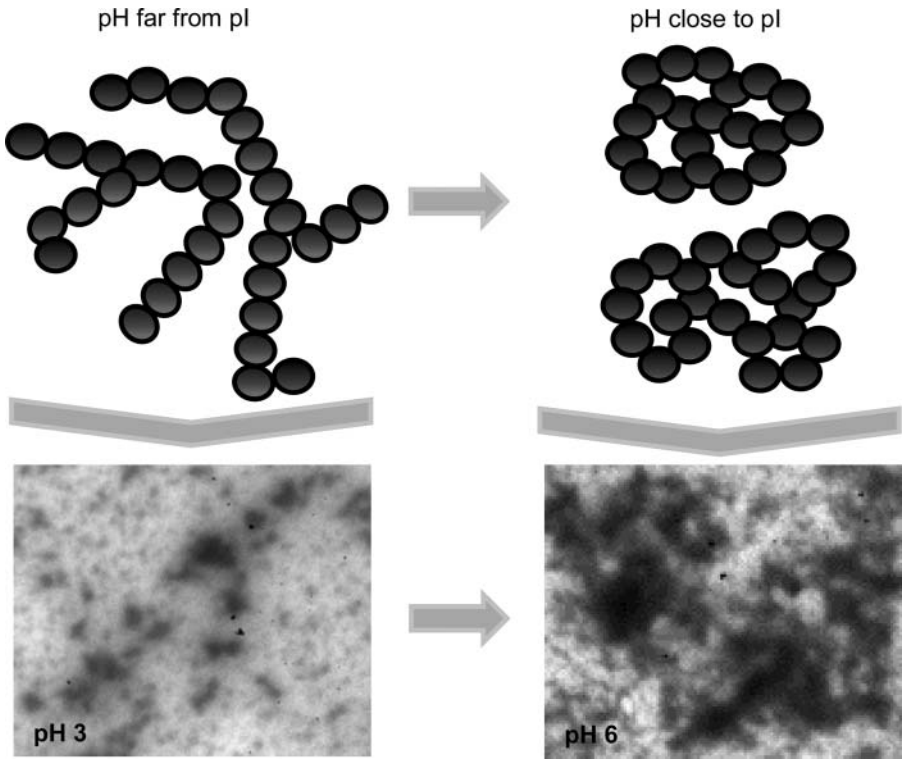

Figure 1. Schematic representation and transmission electron micrographs of filamentous or particulate bovine β-lactoglobulin (10 g L^{-1}) aggregates, formed by heating at 80°C for 20 min under different pH (i.e. 3 and 6) conditions.

proteins, which change charge interactions or even induce different conformational states. This may result in different aggregation behaviors and morphologies of the resulting protein aggregates. The salt type to adjust ionic strength is other condition that should be considered. For instance, calcium and magnesium (divalent ions) can induce aggregation via electrostatic shielding, ion/hydrophobic interactions and cross linking with negatively charged carboxylic groups of neighboring whey protein molecules—leading to the establishment of protein–cation–protein bridges. On the other hand, monovalent cations affect aggregation mainly by reducing repulsions between negatively charged molecules, thus allowing the molecules to come closer to each other giving rise to the formation of noncovalent associations between protein molecules. Different aggregation behaviors can be obtained at the same ionic strength by using different salt type. Divalent ions are more effective at screening electrostatic interactions, thus showing higher ability to form salt bridges and promote aggregation at lower concentrations (Ramos et al., 2012b). The production scheme of a protein nanohydrogel using thermal and salt addition methods to promote gelation is illustrated in Fig. 2; this was the same procedure reported elsewhere (Maltais et al., 2005).

4. Whey protein nanostructures

Gelation of whey proteins toward development of β-Lg hydrogels has been studied extensively during the last decades (Stading and Hermansson, 1990, 1991; Stading et al., 1992; Bryant and McClements, 1998; Kavanagh et al., 2000; Lefèvre and Subirade, 2000; Phan-Xuan et al., 2013). In the following sections, the use of aggregates to produce several kinds of whey protein nanosystems, such as nanohydrogels, nanofibrils, and nanotubes (see Fig. 3), will be reviewed.

4.1. Nanohydrogels

Protein nanohydrogels are three-dimensional, hydrophilic nanosized networks that can swell in water and hold a large amount of water while maintaining the structure due to the presence of covalent and noncovalent bonds, or physical cross-links (Chen et al., 2006; Gyarmati et al., 2013). The swelling capability is the most important feature of a nanohydrogel, and is attributed to the presence of hydrophilic moieties such as hydroxyl, amino and carboxyl groups in the protein structure. This capability makes protein nanohydrogels an ideal structure for encapsulation and delivery of bioactive compounds, and is also responsible for their soft and elastic characteristics (Peppas et al., 2006). The reduced size (sub-cellular) of nanohydrogels coupled with their characteristic structure (large surface area for multivalent bioconjugation, and an interior network for incorporation of nutraceuticals) enables: (i) Controlled release of bioactive compounds; (ii) Improved solubility and bioavailability (especially for those compounds with poor solubility in aqueous matrices or with poor absorption rates); (iii) Specified delivery to the associated tissues, e.g. reducing the gastrointestinal (GI) mucosa irritation caused by continuous contact with some bioactives or protecting them against degradation and undesirable chemical reactions; and (iv) Assured stability of such compounds in the GI tract (Kopeček, 2003; Lin and Metters, 2006; Oh et al., 2009; Tokarev and Minko, 2009; Vermonden et al., 2012). In addition, protein nanohydrogels can be produced easily and designed to spontaneously load biologically active molecules through electrostatic, van-der Waals and/or hydrophobic interactions between the agent and the protein matrix during the gel folding, leading to formation of stable nanostructures in which such compounds become entrapped (Huang et al., 2004; Sahiner et al., 2007; Cerqueira et al., 2014).

Protein nanohydrogels can be prepared from several materials, using different techniques; however, the most commonly used materials are WPI and β-Lg, while gelation is the main technique (Totosaus et al., 2002) – see Table 2. A transmission electron micrograph of β-Lg nanohydrogel produced by physical gelation induced by heating is presented, as an example, in Fig. 3a.

Protein nanohydrogels are able to produce a pre-determined response to the alteration of certain environmental stimuli—e.g. temperature, pH, light, electric or magnetic fields, ionic strength, solvent composition, redox potential or enzymatic conditions, at a desired point and time (Shiga, 1997; Filipcsei et al., 2007; Zhao et al., 2009; Liu and Urban, 2010). These stimuli-sensitive nanohydrogels are of great interest since their

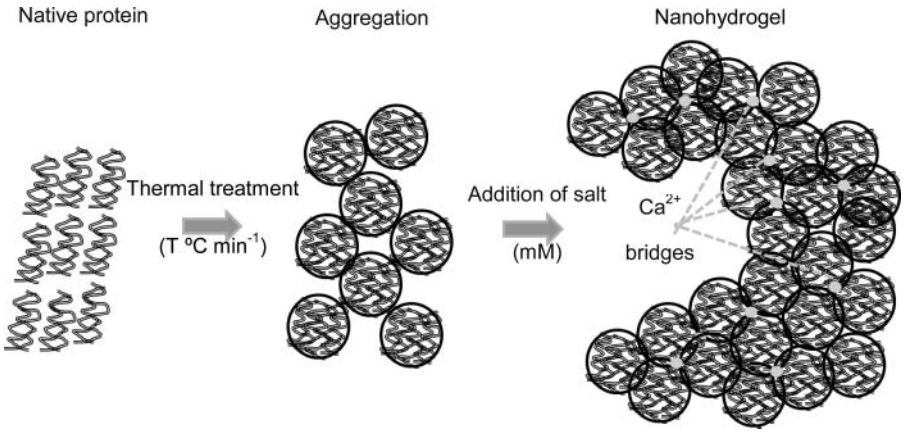

Figure 2. Schematic representation of protein nanohydrogel production through gelation promoted by temperature and salt (Ca^{2+}) addition (adapted from Cerqueira, et al., 2014).

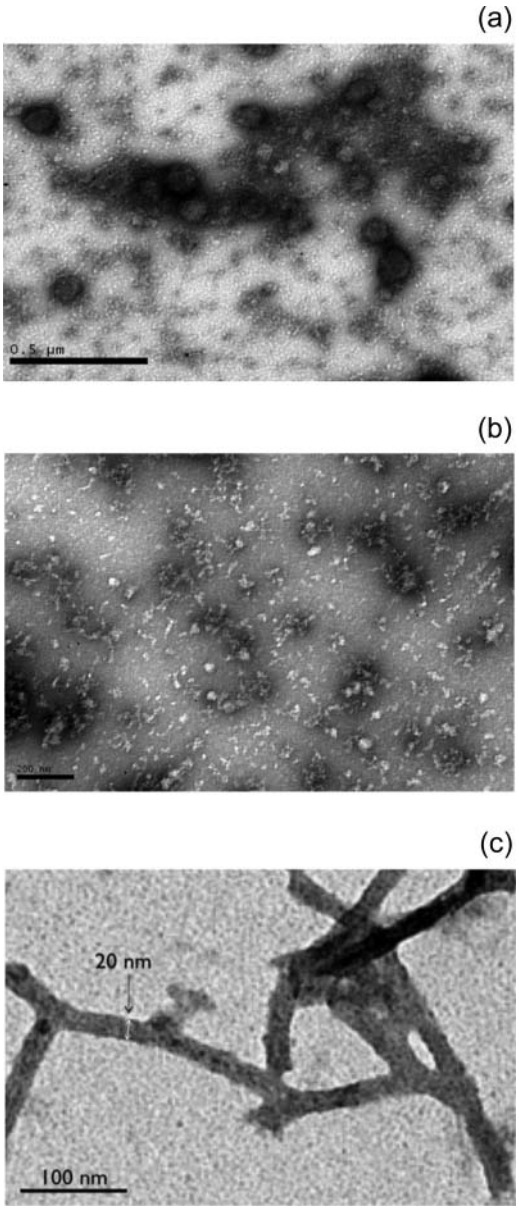

Figure 3. Transmission electron micrographs (negatively stained method) of (a) nanohydrogels and (b) nanofibrils formed from bovine β-lactoglobulin (10 g L^{-1}) by heating at 80°C for 20 min at pH 6.0 and 4.0, respectively; and of (c) nanotubes made from bovine α-Lactalbumin (30 g L^{-1}) partially hydrolyzed with serine endoprotease from *Bacillus licheniformis* (BLP) (4%, w/w), though heating at 50°C for 24 h at pH 7.5, in the presence of manganese.

properties, including swelling/deswelling behaviors and permeability to substances, can be easily and rapidly controlled by external environmental conditions, thus allowing a controlled and specified release of entrapped bioactive compounds to the tissues in the GI tract (Said et al., 2004; Schuetz et al., 2008; Cerqueira et al., 2014).

Due to the aforementioned properties of protein nanohydrogels, these nanosystems are potentially beneficial in biotechnology, and in particular in the food industry as environment-sensitive carriers for bioactive compounds. An additional advantage of this approach is that they can either act as foaming and emulsifying agents toward stabilization of the food (due to their intrinsic viscoelastic properties), or form stable nanocomplexes with other polymers such as polysaccharides—which is a highly desirable characteristic in the manufacture of nanostructures for food applications (Oh et al., 2009).

Zimet and Livney developed a stable nanohydrogel made from a complex of protein-polysaccharide (i.e. β-Lg-Pectin) for encapsulation and delivery of hydrophobic nutraceuticals such as ω-3 fatty acids (DHA) (Zimet and Livney, 2009). Those authors observed that such nanosystems encapsulated efficiently DHA molecules producing a stable system able to protect DHA against oxidation, thus imparting health-improving properties to beverages and food products during storage. Somchue et al. used β-Lg and hen egg white protein as base matrix system for encapsulation of α-tocopherol (Somchue et al., 2009). In order to protect and avoid the release of α-tocopherol under harsh gastric conditions, alginate was used as coating for these encapsulated nanohydrogels. Authors observed that it was possible to protect and maintain the stability of this bioactive compound using a protein based-material. Bengoechea et al. prepared nanohydrogels from bovine lactoferrin by a simple thermal method, able to resist to subsequent pH (from 3 to 11) and salt (from 0 to 200 mM NaCl) alterations, being useful as carriers systems or functional ingredients in food products (Bengoechea et al., 2011). Li et al. designed encapsulation of epigallocatechin-3-gallate (EGCG), the major catechin in green tea and a potent antioxidant, in nanohydrogels of β-Lg (Li et al., 2012). A stable and clear nanosystem was observed at pH 6.4–7.0, and highest protection of EGCG antioxidant activity was obtained with β-Lg heated at 85°C and at the molar ratio of 1:2 (β-Lg:EGCG). In the same way, Shpigelman et al. have nanoentrapped EGCG after cooling and vortexing pre-heated β-Lg solutions (75–85°C, 20 min) (Shpigelman et al., 2010; Shpigelman et al., 2012). The measured association constant with the heated protein was about 3.5-fold higher than that with the native protein. Those authors also found that thermally induced protein-EGCG co-assemblies were smaller than 50 nm, with a zeta potential around−40 mV and a loading efficiency of 60–70% of EGCG within β-Lg nanocomplexes. Limited release of EGCG was observed during simulated gastric digestion of β-Lg–EGCG nanoparticles, suggesting they could potentially be used as vehicles for protection of EGCG in the stomach, and for its sustained release in the intestine. HP was used by Relkin and Shukat as encapsulation technique to entrap α-tocopherol in nanostructures of whey protein dispersions (4 wt.% at pH 6.5), which were previously heated at 65°C, for 5 min (Relkin and Shukat, 2012). Application of a HP step, at 1200 bar, led to decreases in particle charges (to −47 mV) and particle sizes (to 212 nm) accompanied by a more significant destabilization of protein conformation—but only 30% vitamin degradation upon the processing conditions was observed, without further degradation after 8 weeks of storage (Relkin and Shukat, 2012). Recent studies have shown that the size of nanostructures from WPI solutions can be modulated by a combination of desolvation using ethanol, heating and homogenization (Gülseren et al., 2012a, 2012b). These nanostructures were used for zinc entrapment, and exhibited an incorporation efficiency between 80 and 100% (maximum incorporation of about 8 mg g^{-1} WPI). The amount of zinc incorporated in the WPI nanosystem suspensions was within the range of daily zinc requirements for healthy adults, and the particles produced remained stable for 30 days at 22°C and pH 3.0.

Table 2. Whey protein nanosystems and main characteristics: materials and techniques used, encapsulated functional ingredient and other potential applications.

Nanostructure	Material	Technique	Functional ingredient	Applications	Reference
Nanohydrogels	α-La	Thermal gelation	—	Structuring	Doi (1993)
	β-Lg α-La	Heating and cold gelation	—	Structuring	Doi (1993)
	WPI	Thermal gelation	—	Structuring	Puyol et al. (2001)
	WPC	Heating and high pressure	α-Tocopherol	Encapsulation/Delivery	Relkin and Shukat (2012)
	WPI	Heating and ethanol desolvation	Zinc	Encapsulation/Delivery	Gülseren et al. (2012b)
	WPI	Heating and pH cycling	Ethyl hexanoate	Encapsulation/Delivery	Giroux and Britten (2011)
	β-Lg	Heating	Catechin	Encapsulation/Delivery	Shpigelman et al. (2012)
	β-Lg	Thermal gelation	Epigallocatechin-3-gallate	Encapsulation/Delivery	Li et al. (2012)
	β-Lg	Heating and cold gelation	Iron	Encapsulation/Delivery	Remondetto et al. (2004)
Nanofibrils	β-Lg	Extensive heating at low pH	—	Structuring/Nutrition	Bateman et al. (2010)
	β-Lg	Extensive heating at low pH		Structuring/Nutrition	Loveday et al. (2010)
	WPI	Extensive heating at low pH	—	Biosensors	(Esmaeilzadeh et al., 2013; Sasso et al., 2014)
Nanotubes	α-La	Hydrolysis with serine protease	—	Encapsulation/Delivery/ Structuring	Ipsen and Otte, (2007)
	BSA	Layer by layer deposition	Curcumin	Encapsulation/Delivery	Sadeghi et al. (2013)

Note: (–) Note found.

The use of protein nanohydrogels in food applications may bring about some limitations to formulations that contain heat-sensitive ingredients—especially when these nanosystems are produced by thermal gelation. In addition, if these protein structures are produced by physical gelation, they may contain labile bonds in the backbone or in the cross-links that are susceptible of disruption under physiological conditions, either enzymatically (during passage through the GI tract) or chemically, often via hydrolysis (Hennink and van Nostrum, 2002; Hoffman, 2002). Therefore, the ingredients entrapped into such nanostructure can be degraded. The formation of cold-set nanohydrogels may open interesting opportunities for food proteins as carriers of heat-sensitive nutraceutical compounds (Remondetto and Subirade, 2003; Chen et al., 2006), once they can be obtained by adding cationic agents (e.g. ferrous, calcium or barium salts) to solutions of denatured globular proteins. Depending on the protein/cationic agent ratios, different gel network structures can be produced. For example, at lower iron concentrations, filamentous forms can be created by linear aggregation of structural units maintained by hydrophobic interactions, whereas at high iron concentrations, a particulate gel is obtained by random aggregation of large and spherical aggregate units, essentially controlled by van der Waals forces (Remondetto and Subirade, 2003; Sharma, 2012).

4.2. Nanofibrils

Whey proteins possess a great intrinsic propensity to self-assemble into compact three-dimensional structures. The mechanism of self-assembly into fibrils varies, and appears to be specific for each protein (Dobson, 2003; Sagis et al., 2004). Loveday et al. have reviewed the general characteristics of nanofibrils made with several food proteins (Loveday et al., 2009).

When electrostatic repulsions are favored (e.g. pH far from pI and low ionic strength), the whey protein aggregates formed are stabilized by long range, weak attractive interactions, presenting a fibrillar shape. Fig. 3b shows a transmission electron micrograph of nanofibrils formed from bovine β-Lg by heating at 80°C for 20 min at pH 4.0.

In general, whey protein nanofibrils are semi-flexible with persistence lengths larger than one micron, and an average diameter between 4 and 10 nm (Loveday et al., 2012b). Whey nanofibrils can entangle to form physical nanohydrogels at relatively low protein concentrations. Fibril formation may involve several steps, including exposition of hydrophobic regions and preservation of some native surface charges besides reversible formation of linear aggregates, followed by a slow process of "consolidation", after which fibrils no longer disintegrate upon subsequent slow cooling (Arnaudov et al., 2003; Bouhallab and Croguennec, 2014). The fibrillation process can be favored by cleavage of some peptide bonds, and give rise to other supra-molecular structures such as ribbons, spherulites, and nanotubes (Akkermans et al., 2008; Tavares et al., 2014). Ribbons are the result of the lateral stacking of these fibers and are usually obtained after the prolonged heating of globular proteins under acidic conditions (Lara et al., 2011). Alternatively, spherulites are formed by the radial association of the fibers, a structure that can reach hundreds of micrometers in diameter (Domike et al., 2009). The formation of nanotubes from whey proteins is less widespread than the formation of fibers or aggregates, nevertheless, these nanostructures will be discussed below.

Recent research has shown that both pure β-Lg and WPI form nanofibrils upon prolonged heating, at pH below 2.5 and low ionic strength (Gosal et al., 2004; Bolder et al., 2006b; Nicolai et al., 2011). Several studies point at formation of such structures, mostly from β-Lg (Kavanagh et al., 2000; Bolisetty et al., 2012; Dave et al., 2013) and WPI (Ikeda and Morris, 2002; Loveday et al., 2011; Liu and Zhong, 2013). Heating pure α-La or pure BSA at pH 2 is not enough to induce formation of fibrillar structures (Bolder et al., 2006a; Bolder et al., 2006b; Bolder et al., 2007). Other conditions, which may involve salt addition, mild heating and hydrolysis, may be needed to produce α-La or BSA nanofibrils (Goers et al., 2002; Veerman et al., 2003; Loveday et al., 2012a). Few studies exist about the applications of whey protein nanofibrils as nanomaterials. However, it is recognized that these structures may potentially act as thickeners, gelling, emulsifying or foaming ingredients in foods, while also increasing their nutritional value. Recently, other potential applications have attracted a wide interest from food and biomedical industries, such as enzyme immobilization, microencapsulation of bioactive

ingredients, or even development of biosensors (Loveday et al., 2012a; Sasso et al., 2014).

4.3. Nanotubes

Protein nanotubes can be formed through partial hydrolysis of α-La using a serine endoprotease from *Bacillus licheniformis* (also known as BLP or SP-446), in the presence of a divalent cation (Graveland-Bikker et al., 2004; Ipsen and Otte, 2007). A transmission electron micrograph of nanotubes made from bovine α-La (30 g L^{-1}), partially hydrolyzed with BLP, is shown in Fig. 3c.

The formation of α-La nanotubes includes three main steps detailed below and summarized in Fig. 4 (Graveland-Bikker et al., 2004; Otte et al., 2005; Graveland-Bikker and de Kruif, 2006; Ipsen and Otte, 2007; Tarhan et al., 2014):

1. The first step consists on partial hydrolysis of native α-La structure using BLP. This protease induces conformational changes in the resulting peptides, thus allowing formation of β-sheets between two monomers. Consequently, the resulting dimers will form the building blocks necessary for nanotube formation; (ii) The second step occurs once the saturation concentration of dimeric building blocks has been exceeded in the presence of an appropriate divalent cation, e.g. Ca^{2+} (that acts as intermolecular salt-bridge between carboxylic groups on different building blocks). As a result, a stable nucleus is formed consisting in apparently five building blocks; and (iii) The last step includes elongation of the tubular structure through addition of dimeric building blocks to the growing nanotube, in the presence of an appropriate cation. The resulting structure consists of 10-start right-handed helices via β-sheet stacking, with an outer diameter of ca. 20 nm, a cavity diameter ca. 7–8 nm, and several hundreds of nanometers (or even micrometers) long (see Figs. 3c and 4).
2. One of the advantages of gels formed by partial hydrolysis of α-La with BLP will be more translucent and stronger (Ipsen et al., 2001) than those obtained from WPI and β-Lg—which are soft, whitish, and opaque (Otte et al., 1997; Otte et al., 1999; Ipsen et al., 2001). The appearance of α-La gels suggests the formation of strand-shaped structures, thinner than those obtained from β-Lg, which may therefore be more interesting for specific applications. The formation of nanotubes by enzymatic hydrolysis of α-La is influenced by several conditions:
   (i) Type of enzyme: BLP serine endoprotease is specific to peptide bonds containing glutamic (Glu-X) and aspartic (Asp-X) acid residues (Svendsen and Breddam, 1992), and this specificity will determine the tubular shape of α-La nanostructures. α-La has 13 Asp and 7 Glu residues, so if hydrolysis occurs using a protease specific for Glu-X bonds, only 8 building blocks will form, instead of 21 obtained with one specific for both Glu-X and Asp-X bonds, resulting in the formation of disk-shaped nanostructures (Balandrán-Quintana et al., 2013).
   (ii) α-La concentration: the α-La concentration used affects nanotube formation, and therefore elongation rate. Under threshold concentration values, the proteolytic degradation is more extensive, thus resulting in low molecular weight monomers (8.8 kDa), so the saturation concentration of building blocks needed to begin nucleation may never be achieved, assembling into linear and fibrillar aggregates with a diameter ca. 5 nm (Otte et al., 2005). Ipsen and Otte reported that 30 g L^{-1} was the

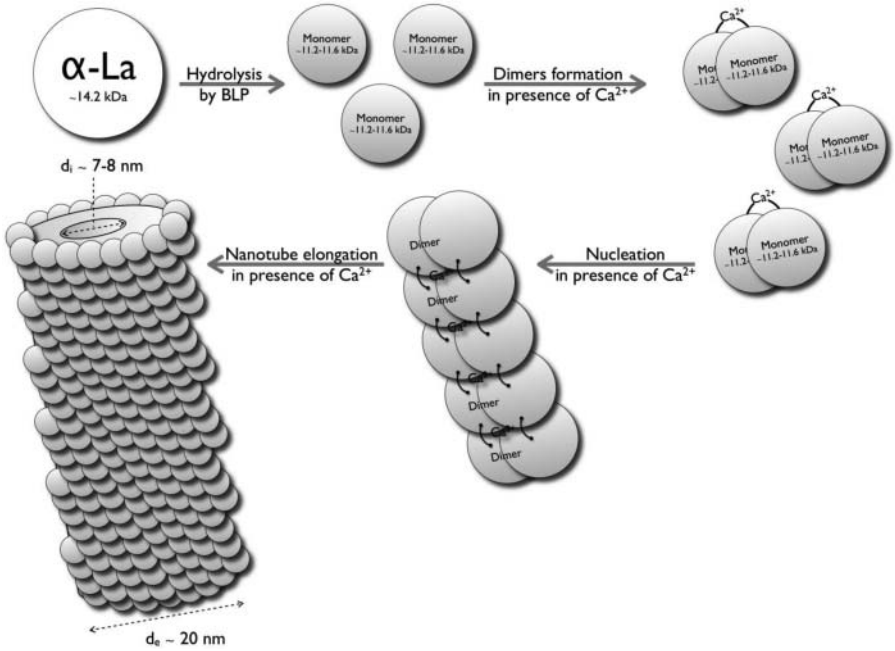

Figure 4. Schematic representation of nanotubes formation from bovine α-lactalbumin (α-La) partially hydrolyzed with serine endoprotease from *Bacillus licheniformis* (BLP), in the presence of calcium (Ca^{2+}) (adapted from Ipsen & Otte, 2007).

threshold concentration of α-La for nanotube formation (Ipsen and Otte, 2007), whereas Graveland-Bikker observed that the nanotubes were also formed at lower concentration of α-La (i.e., 15 and 20 g L^{-1}) (Graveland-Bikker, 2005).

(iii) Type and concentration of cations: Divalent cations play a key factor in formation of nanotubes and its influence depends on concentration. According to Graveland-Bikker the influence of the cation used upon nanotube formation is entirely due to the self-assembly kinetics and not to the enzyme kinetics (Graveland-Bikker et al., 2004). In this work, the author pointed values of Ca^{2+} between 0.5 and 3.0 mol per mol of α-La as threshold concentration for the formation of α-La nanotube. Above and below these values nucleation is too slow, so flocculation and random aggregates formation predominates. Despite Ca^{2+} is the cation most studied for α-La nanotube formation, other divalent cations (i.e. Mn^{2+}, Zn^{2+}, Cu^{2+}, Al^{3+}, Mg^{2+}, and Ba^{2+}) could be used, resulting in structures with different morphologies, optical, and rheological characteristics, probably related to the specific cleaving points for each cation in the α-La. For Zn^{2+}, Cu^{2+} and Al^{3+} the gel formed was stronger and more transparent than that obtained for Mg^{2+} and Ba^{2+}, which result in random aggregates and a weak and turbid gel (Graveland-Bikker et al., 2004).

(iv) Temperature: This is a crucial parameter toward formation of α-La nanotubes, as it impacts on both hydrolysis and nucleation. In the same way, the elongation rate of nanotubes is also temperature-dependent, and increases with temperature (Graveland-Bikker et al., 2004). Temperatures around 70°C, or long heating periods (above 40 min) causes degradation of nanotubular structures resulting in random aggregates (Graveland-Bikker, 2005).

On the other hand, Esmaeilzadeh et al. showed an innovative methodology to synthesize α-La bio-based nanotubes, based on chemical hydrolysis. The nanotubes were produced under application of specific agents (e.g. surfactants, pH reagent, Tris-HCl buffer, and polar solvents) leading to an acid hydrolysis. Therefore, the addition of enzymes or the application of high temperatures are not needed resulting in low cost nanotubes production. In this case, α-La was chemical hydrolyzed through the same cleavage sites used by BLP (13-Asp-X and 7-Glu-X). A divalent cation (e.g. Mn^{2+}, Zn^{2+}, Ca^{2+} and Fe^{2+}) or mixtures are also used to induce tubular self-assembly of partially hydrolyzed α-La and the molar ratio determined growth rate. Nanotubes obtained by this method had 3–8 nm in outer diameters (Esmaeilzadeh et al., 2013).

Protein nanotubes possess several intrinsic advantages over other protein nanostructures, including: (i) Potential of functionalizing the outer and inner layers of nanotubes differently, as both layers have different characteristics; (ii) More efficient delivery and controlled release of bioactive compounds, once they both have open ends; and (iii) High stability, thus maintaining their tubular structure *in vivo* for long periods (Sadeghi et al., 2013).

Despite of the interesting properties of protein nanotubes and their intrinsic potential for use as carrier structures, only one published work describing the application of BSA nanotubes to incorporate curcumin as bioactive compound has appeared—see Table 2 (Sadeghi et al., 2013).

Currently, only carbon nanotubes are commonly used as carriers for controlled release of bioactive molecules, but these are not GRAS materials, mainly due to their potential toxicity for humans. Nanotubes made from α-La might represent a suitable solution for food and health applications, due to such intrinsic characteristics as absence of toxicity, biocompatibility, biodegradability, ease of functionalization and low cost (Ballister et al., 2008; Velusamy and Palaniappan, 2011).

Applications found in the literature for other whey proteins (Graveland-Bikker, 2005; Ipsen and Otte, 2007; Sadeghi et al., 2013; Tavares et al., 2014) and carbon (Feng and Ji, 2011; De Volder et al., 2013) nanotubes can be seen as potential applications for α-La nanotubes, namely: thickener agents, as α-La nanotubes have linear structures highly efficient to improve viscosity; and effective carriers of bioactive compounds, due to gelation capacity of α-La and specified release, due to nanotube ability to disassemble in a controlled manner by lowering the pH at values below pH 3 (Ipsen and Otte, 2007). This behavior could be used to improve the incorporation efficiency and stability of certain nutraceuticals, as well as a controlled release means of such compounds in specified sites of the GI tract. Controlled degradation could also be an advantage when using α-La nanotubes as templates for nanowire synthesis through selective metal deposition, or as scaffolding in tissue engineering (Ipsen and Otte, 2007).

On the other hand, α-La nanotubes exhibit good stability under certain parameters, which make them suitable for industrial applications: α-La nanotubes are sensitive to slight mechanical agitation, but in a reversibly manner (Ipsen and Otte, 2007), and it is also possible to cut them into pieces without damaging their structure (Graveland-Bikker, 2005). α-La nanotubes can also resist short heat treatment equivalent to that normally used for pasteurization of milk (72°C, 40 s) maintaining the integrity of their tubular structure upon freeze-drying and subsequent re-dispersion (Graveland-Bikker, 2005; Ipsen and Otte, 2007).

5. Future trends

Entrapment of bioactives via novel technologies, that may led to high protection of sensitive molecules or to high encapsulation efficiencies constitutes an emerging research area in food industry. The trend is toward a reduction in particle size with particular interest in developing techniques such as electrospraying and electrospinning of whey proteins for the production of nanostructures with improved or novel properties (Tavares et al., 2014).

Recently, considerable progress has been made toward understanding the behavior of several nanostructures in the GI system; however, further work is clearly needed for a full rationalization of whey protein nanohydrogels, nanofibrils, and nanotubes. This information will be crucial to evaluate the biological activity and fate of the ingested whey protein nanosystems and encapsulated bioactive compounds

in vivo, and to ascertain the effects from their use in human health.

For instance, there is little information available about the possible interactions of those nanostructures with components of food and potential effects on toxicity and about their integrity following passage through the digestive system, or how they are absorbed, distributed and excreted from the body (Authority, 2009; Cockburn et al., 2012). The behavior of whey protein nanostructure encapsulating bioactive agents in food products using GI and well-differentiated cell lines (e.g. Caco-2 cells) *in vitro* could be an effective procedure to mimic the characteristics and functions of the intestinal epithelium, in order to address issues such as bioactive compound bioavailability, toxicity and permeability.

At the present stage, a better fundamental understanding of the tract mechanisms of action of whey protein nanostructures at the molecular level will provide a basis for their further optimization and may open more exciting opportunities for their use in the area of bioactive compounds delivery. While a range of *in vitro* screening tests are ongoing, few *in vivo* studies in animals have been carried out, particularly *via* the oral route which is the only relevant route for prediction of risks in food. Therefore, is critical to develop predictive and validated toxicological tests that can be used to screen potential risks, and also to develop new methodology for the measurement of engineered nanomaterials in biological matrices, in order to assess human exposure.

These studies may represent a way to gather new information that address important issues still unresolved, thus contributing to make nanotechnology safer in the coming years for the food industry. Despite the exciting potential of nanotechnology, regulatory authorities and consumers are aware of potential risks arising from extensive use of this technology in food processing (Commission, 2013). Therefore, in addition to toxicological studies, ethical, legal and social issues encompassing food nanotechnology need to be addressed, in order to gain public acceptance (Mody, 2008). Although a number of surveys have examined public understanding and acceptance of nanotechnology, little is known about public perception of nanotechnology use in foods, and even less about use of nanostructures made from food materials (as is the case of whey proteins). The vision of both, the general public and those surveys, appears to be dominated by nonfood examples, especially inorganic materials such as metal nanoparticles and engineered carbon nanotubes.

A recent study conducted by the Food Safety Agency (US) where consumers were asked about the use of nanosystems in food products showed that when they are applied directly to food, consumers are worried; however, their opinion is positive if nanotechnology is used in food products with health benefits (where bioactive compounds can be added) (TNS-BMRB, 2011). It is clear that public perception and consumers' attitudes are the major factors determining the commercial success in this field.

6. Conclusive remarks

Whey proteins are extremely versatile, nutritious and economical food ingredients, and can be used as rich matrices to produce various nanostructures in a number of different ways because of their responsiveness to different environmental factors (e.g. temperature, pH, ionic strength and electric field). These relevant properties make whey proteins promising as building blocks for encapsulation, allowing several associated advantages: (i) No need extended research to prove the safety and noncytotoxicity of co-assembled whey proteins; (ii) Suitable alternative for other nanostructures composed of lipids, which requires the use of organic solvents or surfactants for their manufacture; (iii) High abundant matrix and relative inexpensive to meet the growing demand for additive-free foods; (iv) Controlled disassembly, which is a fundamental step for targeted release; and (iv) Act as carrier agents of bioactive molecules (e.g. hydrophobic vitamins, polyphenols, flavorings, fatty acids, and minerals) for nutraceuticals delivery, and as efficient structuring agents.

Although whey protein nanostructures may be useful in a wide range of consumer food products, there are significant challenges, at present that still remain to be overcome such as: (i) Their large-scale production; (ii) Their assembly and disassembly mechanisms in the presence of bioactives; (iii) Their stability toward processing and storage conditions either on their own or incorporated within food matrices; (iv) Physical and chemical interactions between such nanostructures encapsulating sensitive molecules and complex food matrices; (v) Their robustness and adaptability to harsh conditions during processing and storage and within the GI tract; and (vi) Consensus in the idea that providing confidence to consumers on use of food grade nanosystems in food products implies transparency in the advances attained, and in general more information about risks and benefits regarding their utilization by the food industry.

Overall, the development of whey protein nanosystems for oral delivery of bioactive compounds is been triggering the research from scientific communities and some industrial players, standing itself not only as a high potential solution for some problems faced by the food industry but also as an innovative tool for pharmaceutical applications.

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