Formation and functional properties of protein-polysaccharide electrostatic hydrogels in comparison to protein or polysaccharide hydrogels

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Abstract

Protein and polysaccharide mixed systems have been actively studied for at least 50 years as they can be assembled into functional particles or gels. This article reviews the properties of electrostatic gels, a recently discovered particular case of associative protein-polysaccharide mixtures formed through associative electrostatic interaction under appropriate solution conditions (coupled gel). This review highlights the factors influencing gel formation such as protein-polysaccharide ratio, biopolymer structural characteristics, final pH, ionic strength and total solid concentration. For the first time, the functional properties of protein-polysaccharide coupled gels are presented and discussed in relationship to individual protein and polysaccharide hydrogels. One of their outstanding characteristics is their gel water retention. Up to 600 g of water per g of biopolymer may be retained in the electrostatic gel network compared to a protein gel (3-9 g of water per g of protein). Potential applications of the gels are proposed to enable the food and non-food industries to develop new functional products with desirable attributes or new interesting materials to incorporate bioactive molecules.

Keywords: Protein; Polysaccharide; Mixed electrostatic gel; Gelation Water holding properties; Functional properties.

1. Introduction

Proteins and polysaccharides are classified as biopolymers due to their natural origins and their large polymeric structures. They are commonly used as ingredients in food products for their important roles in the structure and stability of processed foods such as thickening, stabilizing, gelling and emulsifying agents etc. Their simultaneous addition may induce intermolecular interactions offering ways to diversify their functionality. The control of these macromolecular interactions is therefore of high interest for the development of novel food products. For example, proteins and polysaccharides can be processed into functional ingredients to form edible films, to encapsulate vitamins and flavors, to replace fat materials and to form novel semi-solid food products as electrostatic gels [1-4].

When proteins and polysaccharides are mixed together in water, depending on environmental conditions such as pH anionic strength, two different types of interactions can occur: thermodynamic incompatibility also known as segregative phase separation or thermodynamic compatibility resulting in an associative phase separation. Segregative conditions prevail when there is no associative interaction for example between a protein and a neutral polysaccharide or with a polysaccharide wearing charges similar to the protein (as anionic polysaccharide with pH > isoelectric point (Ip) of the protein). More detailed information on protein-polysaccharide segregative systems and their functional properties are discussed in several reviews [5-8]. On the other hand, thermodynamic compatibility is usually induced by associative electrostatic interactions between proteins and polysaccharides when both biopolymers carry net opposite electric charges. These interactions occur at a pH between the proteins' Ip and the polysaccharides' pK_a. Under those conditions, different types of structure can be formed including coacervates, complexes and gels depending on preparation conditions. These structures may be modulated by several factors such as the biopolymers molecular conformation, the charge density and the protein-polysaccharide binding affinity [4].

Coacervates are the result of a phase separation into two liquid phases. The coacervate is found in the phase in which the biopolymers are concentrated while the other phase contains mainly the solvent [9, 10]. Interacting protein and polysaccharide may also form complexes which are aggregates of fractal nature and separate in a phase denser than coacervates. The aggregates properties depend on the protein-polysaccharide ratio. When the protein to polysaccharide ratio allows to reach neutrality of the biopolymer system, a maximum yield of insoluble complex is produced. Soluble complexes may be obtained when the ratio is far from equivalent due to the repulsion between residual charges on the biopolymers [4]. For the interested reader, several reviews on protein-polysaccharide coacervates and complexes detailing the parameters influencing their formation and their functional properties for food applications are available [4, 9, 11-15].

Associative interactions in mixed protein-polysaccharide systems and formation of complexes and coacervates were studied since the nineteen thirties [16]. Ten years ago, the formation of gel under electrostatic associative conditions was first reported for a protein-polysaccharide mixed system [17]. Interaction under quiescent conditions made possible to obtain a gel with a very low solid content (0.03%) without any heat treatment [18]. It was suggested that they may be classified as hydrogel as for each g of biopolymers up to several hundred g of water were retained [19]. Hydrogels are three-dimensional polymeric networks formed by crosslinking polymer chains through physical, ionic or covalent interactions, that can absorb a large amount of water while maintaining their structural integrity [20, 21]. However, the amount of water to be considered as large has not been clearly defined and several authors are using the term hydrogels for any gelled structure containing water which may induce confusion in the interpretation. According to Gulrez and collaborators [22], the terms gels and hydrogels have been used interchangeably by food and biomaterial scientists, respectively.

In this paper, protein based and polysaccharide based gels will be briefly introduced and their gelling conditions will be presented for the purpose of comparison with protein-polysaccharide associative mixed gels. Then, recent progress on the formation and functional properties of protein-polysaccharide electrostatic gels with a particular focus on the effects of the structural characteristics of biopolymers and some environmental factors will be reviewed. Other gelling systems such as synthetic polymer gels are outside the scope of this publication and interested readers are invited to consult other reviews for these types of gelling systems [23, 24].

2. Hydrogels based on protein, polysaccharide and protein-polysaccharide mixtures

2.1 Protein hydrogels

Protein gelation is an important phenomenon to obtain desirable sensory and textural attributes of foods. The gelation of protein has been traditionally achieved by physical treatment (heating, high pressure), enzymatic and chemical treatments (acidification and addition of salt). Most of these gelation methods rely on a mechanism involving unfolding of the native protein structure and aggregation into a gel network that can hold water within its structure. The main protein gelation methods were reviewed by Totosaus et al. [25]. Generally, the protein network is stabilized through non-covalent cross-links such as hydrophobic/electrostatic interactions, hydrogen bonds and/or covalent bonds such as disulfide bonds. The minimal protein concentration needed to form a gel is specific to each protein and it is influenced by their structural characteristics and the gelling conditions (Table 1). Some examples of minimal concentration values are 0.6% for gelatin [26], 3% for egg albumin [26], 6.6% for soy proteins [27] and from 4 to 12% for whey proteins depending on pH and ionic strength [28].

The functional properties of protein hydrogels (gel strength, elasticity, water holding capacity, etc.) depend on the protein intrinsic characteristics, the protein concentration, the ion type and concentration, the pH as well as the processing conditions used to induce gelation

(temperature, time, rate of heating, high pressure treatment, etc.). Globular protein gels have been categorized in fine stranded and particulate gels [29, 30]. The former is a transparent fine-stranded protein hydrogel formed when protein solutions are heated at pH far from protein's Ip with low ionic strength. The latter is obtained at pH close to protein's Ip and/or at high ionic strength, particulate protein hydrogels are then formed. This behavior has been reported for whey proteins [29-31], egg proteins [32] and other globular proteins [33]. The particulate hydrogels are coarser, opaque, weak and brittle and retain less water in their structure after centrifugation compared to a fine stranded protein gel [34, 35]. Additional information on protein hydrogels properties are presented in section 6. More details on formation, structure and applications of protein gels can be found in several publications [36-40]

2.2 Polysaccharide hydrogels

Polysaccharide with their molecular weight ranging from several hundred thousand Daltons to millions of Daltons through various intermolecular interactions allow gel formation at concentrations lower than 1% [41] lower values than the one required for protein gelation (Table 1). Several factors influence polysaccharides gelation. Molecular characteristics as the molecular weight, the monosaccharide composition, the charge density (sulfate/carboxylic groups) and the conformation (flexibility) are known important factors. Extrinsic factors as the temperature, the presence of specific counter ions and/or the pH also modulate polysaccharide gelation. Variation in some of these extrinsic factors may provoke changes in the polysaccharide conformation from a disordered to an ordered state [42]. Intermolecular associations between ordered domains form physical crosslinks of the network entrapping water. The driving force for cross-linking varies between polysaccharides and each has a specific gelation mechanism and concentrations needed to form a gel. For example, the aggregation and network formation are driven by hydrogen bonds for agar gelation while ionic interactions are mainly involved in the gelation process of alginate [43, 44] or low-methoxyl

pectin (LM-pectin) [45] and both types of interactions explain carrageenan gelation [46]. The concentrations required for gelation vary between polysaccharides. Concentrations lower than 1 % for agar gels [47] and 0.7 wt% for κ-carrageenan gels [48] were found. Gel concentrations ranging from 0.5-2% were reported for alginate depending on calcium concentration and alginate sources [49]. Some polysaccharides are non-gelling due to conformational restriction or repulsive conditions hindering gel formation. Xanthan gum is considered as a non-gelling polysaccharide [50] and only the presence of divalent or trivalent metal ions allowed a sol-gel transition [51, 52] Similarly, λ-carrageenan is a non-gelling polysaccharide mainly due to the presence of three sulfate groups per disaccharide units causing repulsive conditions [41]. The wide range of polysaccharide structures and gelling conditions and their resulting gel properties offer opportunities to create mixed gels with tailor made attributes.

2.3 Protein-polysaccharide hydrogels

Proteins and polysaccharides are often used simultaneously to control the structure, the texture and the stability of food products [5, 8, 12, 53, 54]. These mixtures could provide a gelling system with different types of gel structures depending on the characteristics of the biopolymers used and the environmental conditions. Interpenetrating networks, phase-separated networks and coupled gels could be obtained when at least one biopolymer may form a gel [55, 56]. A typical interpenetrating network has been observed in bovine serum albumin (BSA)/LM-pectin mixtures if calcium ions were present. A very weak protein aggregate network formed by heating was interpenetrated by a LM-pectin network formed with calcium ions upon cooling [57]. Therefore, both biopolymers form two independent gelled networks.

Phase-separated networks are obtained under segregative conditions when some degree of demixing between protein and polysaccharides phases occurs prior to gelation. The process of phase separation is stopped when gelation occurs. The relative rates of phase separation and

gelation determine the final structure [58]. The balance between phase separation and gelation depends mainly on the electrical charges of the biopolymers which are modulated by factors such as the ionic strength [59], the pH [53, 60, 61] and on the heating/cooling kinetics [62]. When heating rates are slow, the time needed to reach the temperature of protein denaturation is longer and the phase separation is enhanced compared to a faster heating rate. The separated phase containing protein is then found as included large spherical areas compared to a faster heating rate where protein phase is dispersed in small spherical entities. Moreover, for a system with a constant heating rate, as pH moves away from Ip, stronger repulsion conditions resulted in more extended phase separation. Large protein zones were formed preceding gelation as compared to lower pH conditions in which protein microgel sizes were smaller [59, 62]. Similarly, whey protein in mixture with polysaccharide of varying charge density permit to modulate gel structure and its properties through the extent of phase separation [63, 64]. Phase separation imply an increased concentration in the protein phase and this contributes to gel formation at a lower concentration in a mixed gel as compared to a protein gel [65]. It was possible to form a gel at 8% protein content at pH 6 with addition of 1% pectin while protein alone did not gel [63]. Gel strength and water holding capacity of whey protein-pectin gels were also improved compared to proteins gels. Numerous mixed protein-polysaccharide systems with phase separated gels were studied in the last three decades and readers are referred to the following reviews [1, 5, 6, 8, 53, 66].

Coupled gel networks are formed under associative conditions when two biopolymers are linked together through junction zones. This type of gels has been associated to the synergistic effect observed in some mixtures of two polysaccharides as xanthan gum and galactomannan [67]. However, only few studies have reported the formation of coupled gels in protein-polysaccharide mixtures [17, 68]. Complex and coacervate structures are most frequently obtained in protein-polysaccharide mixtures under associative conditions and quiescent

conditions are required to reach a gel state [4]. A coupled gel was first reported for a mixture of β-lactoglobulin and xanthan gum [17] and further investigations showed the same behavior for several other protein-polysaccharide systems (for example: caseinate, bovine serum albumin, lysozyme with xanthan gum, gellan gum, λ-carrageenan; see section 4.2) [18, 69]. The minimum biopolymer concentration needed for gel formation varied from 0.03-1 wt% depending on biopolymer mixtures.

3. Mechanism of protein-polysaccharide electrostatic gel formation

Since the first report of gelation under electrostatic associative conditions in protein-polysaccharide system, several methods were used to characterize their structure and properties allowing to propose a gelation mechanism (Fig. 1) [17, 19, 70]. Polysaccharides in aqueous solution adopt an extended conformation to reduce electrostatic repulsion and consequently entropy. In the case of xanthan gum, this polysaccharide exhibits a pseudoplastic behavior due to xanthan gum molecules end-to-end association [71] or side–side association [72], which result in a tenuous network of xanthan gum molecules in solution. From light scattering, rheological and confocal microscopy results, it was proposed that the gelation kinetic of β-lactoglobulin-xanthan gum mixture occurred in three stages (denoted as I, II and III in Fig. 1) [70]. During acidification, as the pH approaches the Ip of the protein, the interaction between positively charged patches on the β-lactoglobulin with negatively charged groups (COO^-) on xanthan gum chains results in the formation of soluble complexes (stage I, Fig. 1). With further pH decrease, more protein aggregates on xanthan gum chains and the net charge of soluble complexes is reduced. Soluble complexes aggregate into interpolymer complexes (stage II) due to the formation of junction zones where two xanthan gum chains might share the same protein molecules (see the arrows in Fig. 1). The association that occurs as electrostatic associative interactions increase results in a sol-gel transition at the point of gelation (stage III). The network of xanthan gum chains provided a frame for gel organization and β-lactoglobulin aggregated

along the xanthan gum chains and therefore may be regarded as a crosslinking agent. At high β-lactoglobulin-xanthan gum ratios, the gels may have multiple layers of proteins that aggregate along xanthan gum chains because larger strands were observed in the gel structures obtained using confocal laser scanning microscope [70]. β-lactoglobulin-xanthan gum system has been mostly studied, but similar arguments are valuable for electrostatic gelation of other protein-polysaccharide mixtures.

4. Factors influencing electrostatic gelation and gel properties

The primary driving force for the association of proteins and polysaccharides in aqueous solutions is electrostatic interactions [2, 17, 18, 70, 73]. Hence, the formation of protein-polysaccharide electrostatic gels is influenced by several factors (Fig. 2). These factors can be classified as factors modulating electrostatic interactions through the overall charge in the mixed systems and factors impacting the network backbone structure. Electrostatic interactions depends on environmental factors (pH and ionic strength), biopolymer charge density and ratio. The network backbone structure is defined by the nature and the characteristic of each interacting molecules (molecular weight and flexibility) [4, 13] and biopolymer concentration. The main factors will be discussed in the next sections.

4.1 Shear conditions

Gel formation of associative protein-polysaccharide system is possible only if the acidification is performed without agitation of the solution (quiescent conditions) [17]. The biopolymers can then interact progressively as the charges are gradually modified during acidification. Glucono-δ-lactone (GDL) is used as acidifier. GDL is hydrolyzed to gluconic acid and thus, allows gradual and homogeneous lowering of the pH throughout the solution. Acidification using other types of acid requires mixing and this induces protein and polysaccharide structural reorganization and complexes formation instead of the formation of a three-dimensional gelled

network. Quiescent conditions during acidification is then a prerequisite to obtain a gel. It should be noted that when the pH of mixture has to be increased to reach a pH > Ip to allow protein interaction with a cationic polysaccharide like chitosan, a basic compound should be used as a pH modifying agent to increase gradually the pH (ex. sodium aluminum phosphate) [18].

4.2 Biopolymer nature and characteristics

Protein and polysaccharides structural characteristics affect the gel formation and properties (Table 2 and 2). Both xanthan gum and λ-carrageenan, two non-gelling polysaccharides, were able to form a gel in combination with proteins (Table 2). Independently of the protein source, higher concentration in biopolymers was needed for λ-carrageenan compared to xanthan gum and it was attributed to their different structural characteristics. The higher molecular weight and intermolecular association tendency of xanthan gum (>1000 kDa) at rest may facilitate gel formation through associative interactions with proteins during acidification [56, 74]. In addition to its smaller molecular weight (300-600 kDa) the higher charge density of λ-carrageenan (3 sulfate groups per disaccharide) induces more entropy in solution and may increase the concentration necessary to reach an ordered gelled structure [41]. Gellan gum, another bacterial polysaccharide, allowed to form a gel at intermediate concentration between xanthan gum and λ-carrageenan. The acyl type gellan gum used is an unbranched, doubled helix and stiff polymer chain that may form demoldable gels at concentration as low as 0.05 wt%[75]. In the condition studied, gellan gum alone did not form a gel (results not shown) but in mixture with protein, a gel was obtained within concentration ranging from 0.07-0.65 wt%. It should also be considered that the ratio used for comparison (ratio 2) may not be the optimal ratio for the mixed systems studied as this ratio was 3.5 for β-lactoglobulin-xanthan gum [17, 70]. In summary, polysaccharide concentration in mixed gelled systems ranged from 0.01-0.36 wt% (Table 2) while for polysaccharide gels higher concentration were reported (> 0.7 wt%, section 2.2).

Moreover, non-gelling xanthan gum and λ-carrageenan were able to form mixed gels showing the potential of this gelation process to develop new applications.

The protein used in the coupled gel formation also influenced the minimal total solid concentration to achieve gel formation. When β-lactoglobulin and BSA (both globular proteins) were used to form electrostatic gels with xanthan gum and gellan gum; firmer gels were obtained with BSA and lower biopolymer concentrations were required to reach the gel point (Table 2). This observation may be explained by the higher charge density of BSA compared to β-lactoglobulin and also its higher molecular weight (BSA: 66.43 kDa vs β-lactoglobulin: 18.4 kDa) [76, 77]. Therefore, BSA has more reactive sites which may promote stronger electrostatic interactions with the polysaccharides. In that case, excessive associative interactions caused spontaneous syneresis and water was expelled from the gel structure [18]. Caseinates with a disordered protein structure also contributed to gel formation in these coupled gels but higher concentrations were required.

The addition of salt, before gel formation, resulted in an increase in the concentration needed to form a gel for xanthan gum and λ-carrageenan but the opposite behavior was found for gellan gum (Table 2). The addition of salt may impact gel formation in two ways: by changing the biopolymer flexibility and by screening of the charged reactive sites reducing electrostatic associative interactions between protein and polysaccharide. This happened for xanthan gum and λ-carrageenan mixed gels. In the case of gellan gum, salt probably induced a reduction of repulsive interactions between gellan gum molecules favoring intermolecular aggregation at lower concentration [78]. The effect of salt on gel strength will be presented in section 4.3.

In addition to the critical concentration to form a gel, the conformation of biopolymers may also impact some gel properties as the gel strength of protein-polysaccharide gel systems (Table 3).

When longer and stiffer polysaccharides were used, the gels were more elastic with higher final G' values; e.g. xanthan gum > gellan gum > λ-carrageenan and alginate (Table 3). This is in accordance with the lower gel critical concentration for xanthan and gellan gums compared to carrageenan (Table 2). Alginate did not allow gel formation (G' < 1) but complexes were formed even under quiescent conditions (Table 3). Until recently all the systems studied were made of mixtures with proteins having an Ip below pH 5 and consequently, were forming gel in acidic pH conditions only. It is possible to form a coupled gel with a final pH around 7 using basic proteins (example: lysozyme Ip = 10.7) and an anionic polysaccharide [69]. Gels obtained with lysozyme showed lower G' compared to the β-lactoglobulin-xanthan gum system (76 vs 342 Pa). This lower G' value may be due to different protein properties but it should be also considered that at pH 7, the residual charge in the lysozyme-xanthan gum mixture is -20 mV compared to -50 mV for β-lactoglobulin-xanthan gum. For each set of protein and polysaccharide, there are optimal conditions (pH, ratio) to ensure good gel properties and this will be further discussed in the next section.

4.3 Protein-to-polysaccharide ratio and biopolymer concentration

The protein-to-polysaccharide ratio is critical to control the charge balance between interacting biopolymers [73, 79] and therefore, imparts the number of protein molecules bound to a polysaccharide molecule. For a specific protein-polysaccharide pair, there is an optimal ratio for which electrostatic interactions reach an equilibrium between repulsive and associative interactions allowing the formation of the strongest gel at a specific pH. This ratio was found at 3.5 for β-lactoglobulin-xanthan gum system at a final pH of 4.4 [70]. The ratio is then the driving parameter of gel structure and properties. From this optimal ratio, increasing the protein content (higher ratio) decreased the elastic modulus (Fig 3A), hardness at gel fracture point and gels were more opaque [17, 19, 70]. As the protein-polysaccharide ratio decreases from 10 to 2, the gel density has increased characterized by a smaller network pore size and subsequently, gel

304 water retention is improved (Fig. 3B). A linear relationship between gel porosity and syneresis
305 exists. Larger pore size is associated to higher syneresis values. For a specific ratio, it is
306 possible to control the gel properties by increasing the biopolymer concentration. The gel
307 strength increases with concentration while the pore size and syneresis of the gel are reduced
308 (Fig. 3). This is not attributed to the gel final pH as it depends mainly on the ratio [70]. The large
309 deformation behaviour is also affected by concentration as the hardness (force to reach fracture
310 point) increases with higher concentrations [19] and this is consistent with a previous report for
311 protein networks [80].
312
313 *4.4 Ionic strength*
314 Factors affecting the ionic strength of solution as the presence of minerals and addition of salt,
315 are expected to impact the electrostatic gel properties. Salt addition (20 to 50 mM NaCl) had a
316 strong effect on the gelation of β-lactoglobulin-xanthan gum mixture and gel properties (Table
317 4). The gelation rate (dG'/dpH) was slowed with 20 mM NaCl and the gelation process was even
318 prevented with addition of 50mM NaCl, resulting in electrostatic precipitated complexes instead
319 of an organized gel network [17, 70]. At 20 mM of NaCl, a more open network with higher pore
320 size (6.7 µm) than the gel without salt was obtained corresponding to a lower final G'.
321 Consequently, the gel network had lower water holding capacity and higher syneresis values.
322 The effect of NaCl is explained by the shielding of charged reactive groups on proteins and
323 polysaccharides weakening the network structure. It may also impact the entanglement of
324 xanthan gum chains [81] and therefore its ability to form a coupled gel with protein.
325
326 **5. Stability of electrostatic gels**
327 Electrostatic protein-polysaccharide mixed gels are pH-reversible, inherent to the nature of
328 interactions involved in their formation. If suspended in a phosphate buffer at pH 7 above the Ip
329 of the protein or at a pH below the pKa of the polysaccharide, the gels liquefy rapidly [70]. As

presented previously, the gel is also weakened with salt addition. The instability of these gels to pH and salt is a limitation for their applications in the food industry. The same challenge exists for electrostatic complexes and an heat treatment has been proposed as a stabilisation process to induce the formation of additional bonds (covalent, hydrophobic, etc) [2, 82]. According to these authors, whey protein–pectin complexes were stabilized with heating conditions (85 °C x 15 min and 90 °C x 2 min). A similar heat treatment (80 °C, 30 min) has been successfully applied to stabilize electrostatic gels [83]. The stability in phosphate buffer at pH 7 was dependent on the β-lactoglobulin-xanthan gum ratio. At ratio of 2, the heated gel was completely melted after 30 min soaking time. However, at ratio of 20, the gel did not melt due to increased stability after heat treatment. In addition, the stabilization process has increased the storage modulus (G') of the heated gel (ratio 5: 72-382 Pa). Heating the coupled gel did not modify the overall structure of the network as the pore size was unchanged. The gel network has been strengthened by the accumulation of protein on the initial network backbone after heat treatment as revealed by a brighter network branches as observed in confocal microscopy (Fig. 4D vs 4A). However, the polysaccharide network did not change upon heating (Fig. 4B vs E). Additional work is needed to determine the optimal heating conditions allowing stabilization of these electrostatic gels to ensure widespread uses.

6. Electrostatic gel functional properties

Electrostatic gels are generally formed in conditions under which protein and polysaccharide would not individually form gels. It is difficult to compare electrostatic mixed gel properties with previous work performed on proteins or polysaccharides as the gelling conditions and techniques used vary widely in the literature. Consequently, in this review paper no attempt was made to link the rheological behaviour of those systems. However, some recent papers investigating the water holding properties of various globular proteins gels [35, 84-86] used

similar methodological approach [87] allowing comparison with electrostatic gels. Therefore, comparison of protein and electrostatic gels will be outlined next using water holding properties.

The most distinctive characteristic of the electrostatic protein-polysaccharide hydrogels compared to individual protein or polysaccharide hydrogels is their ability to form a gel at very low concentration without heat treatment and their exceptional ability to entrap water. Water holding properties of protein gels are determined by both microstructure [84] and gel stiffness (resistance to deformation of the gel) [85]. It was proposed that water holding is the sum of several contributions as molecularly bound water (0.06-0.07g water/g of globular protein), water captured in the structure of the protein aggregates building blocks (at the submicron level) and water included in the structure at the micrometer level (also referring to porosity, connectivity) [84]. These authors associated water loss to length scales where inhomogeneities were observed in the protein gels (Table 5). Gel structure and water holding properties of some heat-induced protein gels and electrostatic induced gels are compared in Table 5. The length scale at which water loss is apparent is in the micrometer range and is specific to each protein system and gelling conditions. Ovalbumin gels formed at pH 7.5 changed from fine stranded to particulate types of gel with salt addition and length scales associated with the loss of water retention vary from 0.1-0.4 µm. Whey proteins fine stranded gels also showed inhomogeneities at smaller length scales (0.03 µm) than systems with salt (2 µm) for which syneresis is increased by 10 fold. Modification of soy protein with succinyl groups allowed to reduce the coarsening effect of calcium on gels [86]. This has also improved water retention and held water in the gel (9.0 g water per g protein for succynilated protein vs 4 for native soy protein). In summary, these different heated proteins were able to hold from 3-9 g water/g protein, consistent with previous studies [87]. In comparison, electrostatic induced gels showed much higher values, up to several hundreds g of water/g biopolymers (Table 5). As seen previously, the total solid concentration and the ratio significantly impact syneresis (Figure 3B) and

therefore held-water. Gels produced at acidic (β-lactoglobulin-xanthan gum) and neutral (lysozyme-xanthan gum) pH both exhibit similar water holding properties associated with equivalent gel pore sizes (3.8–4.7 μm). Furthermore, the gel which has been heat-stabilized showed similar gel pore size (2.7–2.9 μm) and water holding properties (Table 5). No coarseness was observed in the electrostatic gel but the pore size was inversely related to held water; larger pores being less efficient to retain water. Mixed gel's pore size is larger than protein gel pore size suggesting a physical entrapment of water in the network. Systems formed at very low solid contents are then easily breakable. Under shear, gel are broken and biopolymers are found in a separated phase containing precipitated complexes.

The relationship between pore size and water holding properties is still mainly descriptive and the role of some factors such as gel coarseness, heterogeneity and biopolymer type is still not well understood. For example, capillarity has been proposed as the prevailing hypothesis for water retention by Stevenson who considered hydrogels has 3D interconnected capillary tubes. Consequently, according to Young-Laplace equation, as pore size increases capillary pressure (in a capillary tube) decreases simultaneously with water holding capacity [88, 89]. The use of methods measuring water holding based on centrifugation an external pressure equivalent to the capillary pressure will be necessary to remove water from the gel [90]. Compared to protein hydrogels, mixed gels have larger pore size and water is easily expulsed out of the gel in accordance with the capillarity hypothesis. Therefore, for a specific gel network, factors (concentration, ratio, biopolymer type) contributing to reduce pore size will improve their water holding properties.

7. Potential applications of electrostatic gels and microgels

The electrostatic protein-polysaccharide gels clearly qualify as hydrogels, with their high water content (up to 600 g water per g biopolymer, ratio 2, 0.1 wt%). As reviewed, several factors can

be used to leverage the gel properties. For example, water retention is driven by the biopolymer source, ratio, concentration, final pH, etc. The term hydrogel applied to cross-linked macromolecular network was introduced more than 50 years ago. For several decades, research projects were launched to tailor made hydrogels for various purposes as solute diffusivity, mechanical properties, etc [91]. Since then, the second generation of hydrogels were designed to be responsive to environmental changes as the pH, temperature or concentration and it was the premise to the development of smart hydrogels (formed *in situ*, with desired release kinetics, etc.). Initially made of synthetic polymer, the use of natural biopolymers is becoming of increased interest [3]. The protein-polysaccharide hydrogels are based on natural sources of biopolymers and being formed without heat treatment, they can be considered as promising natural hydrogels. The electrostatic nature of stabilizing interactions make these gels responsive to pH changes and temperature. For example, salt addition increases pore size in electrostatic gels (see section 4.4). Mixed electrostatic hydrogels may find applications in areas similar to synthetic polymer hydrogels as encapsulation and delivery systems in non-food applications. In addition, the fabrication process without heat treatment is particularly well adapted to protect sensitive bioactives. Uses in food may be broad but are limited by the gel formation process and its pH stability. However, fundamental knowledge on the electrostatic mixed gel formation and properties may support the thoughtful use of exopolysaccharides producing strain in yogurt. Some strains are producing anionic exopolysaccharide capable of interacting with the protein network during gel formation modifying rheological properties [92, 93]. It may be hypothesized that similar network formation and functionality (high water retention) of the electrostatic gels reported in this review may be involved in these systems as well.

Once stabilized by heat treatment, applicability of these type of gels is wider. Hydrogels may be further processed using shear treatment to produce microgels, also referred as "broken gels"

[3]. Microgels are colloidal dispersions of gel-like particles [94] with promising properties [95]. Microgel size and morphology depend on the type of equipment used and the shear treatment applied. Traditional microgel fabrication are based on emulsion gelation while the sheared gel process eliminates the need to separate microgels from oil and is easily upscaled [3]. The processing of hydrogels into microgels allows to expand their potential uses in food systems. Protein-polysaccharides microgels could contribute to increase water retention in the food matrix and to improve their rheological and textural effects. They also can serve as encapsulation and delivery system. Protein microgels were also proposed as alternative colloidal ingredients for the stabilization of food emulsions [95] and foams [96]. Electrostatic microgels could also be valuable for these applications based on their amphiphilic character due to the protein content. Microgels, could also replace fat in order to mimic some of the desirable characteristics of lipid droplets such as the appearance, mouth feel and texture [2]. The wide range of water holding capacity of electrostatic gels may also offer new flavor delivery possibilities and may control food juiciness [97]. In the next years, more work is needed to validate microgels formation from electrostatic protein-polysaccharide hydrogels and to determine their functionality in various food systems.

8. Conclusions

Hydrogels made of protein-polysaccharide interacting through electrostatic interactions may be produced from several natural biopolymers. They are obtained through a gelation method not requiring any denaturing step as opposed to protein hydrogel. Porous gels entrapping large amounts of water are obtained at low concentration. Modulation of hydrogels porosity and functionality is possible through fabrication conditions (choice of biopolymer, concentration, protein-polysaccharide ratio, pH and salt content). Mixed hydrogels were compared to other largely used protein hydrogels showing that electrostatic gels may be obtained at significantly lower concentrations and were more efficient to entrap water. Stabilization of these hydrogels

has been proposed to enlarge their uses and to allow their further processing in microgels. These microgels have the potential to act as rheological modifiers and emulsion stabilizer properties.

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Table 1. Proteins and polysaccharides structure and properties.

Polymers	Molecular weight kDa	Flexibility/ conformation	Pk_a/Ip	Critical gel concentration	Parameters control gel characteristics	Ref.
Proteins						
β-lactoglobulin	18.4	Globular		Heating ramp up to 85°C: 1 wt% (pH 4.4-5.5) ~ 5 wt% (pH < 4) > 10 wt% (pH > 6)	Protein concentration, heating rate and pH.	[98]
Bovine Serum Albumin (BSA)	66.4	Globular	4.7-4.9	4 wt% (90°C x 45 min, pH 8)	Heating temperature and time, protein concentration, pH, salt.	[99]
Lysozyme	14.3	Globular	10.7	~ 4.3 wt% (20 mM DTT, 85°C x 10 min)	Lysozyme concentration	[100]
Polysaccharides						
Alginate	150-1700	Unbranched, extended random coil	3.38 to 3.65[1]	0.5-2 wt% (30 mM GDL, 15 mM $CaCO_3$)	Alginate Mw and concentration, proportion of mannuronic and guluronic acid residues	[49]
Carrageenan	300-600	Unbranched, semiflexible polymer chain	~4.3	0.5-3 wt%	Type of cations, carrageenan source	[101]
Low acyl-gellan gum	200-300	Unbranched, double helix, stiff polymer chain	~3.6	0.05 wt% (low sugar content, pH 3.5-6.5)	pH, sugar and cations concentration.	[75]
Xanthan gum (XG)	2000	Branched, double helix, stiff polymer chain	~2.8	No gel formation	-	[50]

[1] Refers to the pKa of mannuronic and guluronic acid respectively.

Table 2. The minimum total solid concentration of biopolymers for gel formation of several protein-polysaccharide systems at ratio 2.[a]

	Xanthan Gum		λ-Carrageenan		Gellan Gum	
	NaCl addition		NaCl addition		NaCl addition	
	0 M	0.02 M	0 M	0.02 M	0 M	0.02 M
WPI (80 % protein)	0.04	0.10	0.90	nd	0.07	0.05
β-lactoglobulin	0.04	0.15	0.40	nd	0.09	0.06
Na-Caseinate	0.05	0.15	1.10	nd	0.65	0.15
Ca-Caseinate	0.05	0.12	0.90	nd	0.25	0.12
Bovin serum albumin (BSA)	0.03	0.04	0.40	nd	0.09	0.04

[a]Adapted from Laneuville, Turgeon [18].

The gelation was performed in tubes with GDL addition to reach a final pH of 4.6. Values are the minimum total solid concentration of biopolymers at which the gel is not disrupted by inversion of the tube.

nd: No gel were formed at biopolymer concentration below 1.2 wt%.

Table 3. Effect of biopolymers on final storage modulus of electrostatic gels.

Proteins sources	Gelling conditions			Final G' (Pa)			
	Ratio	Total solid wt%	Final pH	Xanthan Gum	Gellan Gum	λ-Carrageenan	Alginate
β-lactoglobulin [a]	2	0.5	3.4-4	3388 ± 200	646 ± 37	5 ± 3	0.8 ± 0.3
β-lactoglobulin [b]	0.5	0.3	4.1	342 ± 52	NA	NA	NA
Lysozyme [b]	0.5	0.3	7	76 ± 1	NA	NA	NA

NA: not analyzed.

[a] Unpublished results

[b] Adapted from de Souza [69].

Table 4. Effect of salt addition on gel properties obtained from mixture of β-lactoglobulin-xanthan gum.

NaCl	dG'/dpH [a]	Final G' [b] Pa	Pore size [c] µm	Syneresis [d] %
0 mM	206	396 ± 7	2.7 ± 0.1	29.2 ± 0.8
20 mM	64	100 ± 16	6.7 ± 0.4	90.4 ± 1.2
50 mM	nd	8 ± 3	nd	nd

The mixture of β-lactoglobulin-xanthan gum had a total solid concentration of 0.36 wt% and ratio of 5. Adapted from Le [83].

[a] dG'/dpH: Gelation rate, meaning the increase of G' with pH reduction, calculated as the average of $\Delta G/\Delta pH$ for 5 consecutive measurements, the first measurement was taken at the gelation point when the G' value rose above 1 Pa.

[b] Final storage modulus of gel at pH 4.4.

[c] Pore size : Average diameter estimated by image analysis of confocal micrographs using ImageJ [19].

[d] Syneresis was measured after centrifugation (120 g x 4 min) [19].

nd: not determined due to formation of complexes instead of gels.

Table 5: Water holding properties of biopolymer gels in relation with gel coarseness (length scales or gel pore size).

Gel types and gelling conditions	Length scales[a] µm	Pore size[b] µm	Syneresis %	Held-water g water/g biopolymer	Centrifugation parameters[d]	Ref.
Heat-induced protein gels						
Ovalbumin gels (12 wt%, pH 7.5, 95 °C, 20 min)					100 kPa, 10 min	[85]
0 mM NaCl	0.1		5 [c]	7 [c]		
300 mM NaCl	0.4	nd	55 [c]	3 [c]		
Whey protein isolate gels (14 wt%, pH 7.2, 95 °C, 30 min)					300 kPa, 10 min	[35]
0 mM NaCl	0.03		5 [c]	6 [c]		
300 mM NaCl	2.00	nd	50 [c]	3 [c]		
Soy protein gels (10 wt%, 100 mM $CaCl_2$, pH 7.0, 95 °C, 30 min)					20 kPa, 10 min	[86]
0% degree of succinylation	4.7		60 [c]	4 [c]		
73% degree of succinylation	0.2	nd	0 [c]	9 [c]		
Electrostatic-induced gels						
β-lactoglobulin-xanthan gum gels (final pH 4.4, 25 °C)					14.52 kPa [e], 4 min	[19, 69]
0.3 wt% ratio 0.5		3.8	3	321		
0.36 wt% ratio 5	nd	2.7	29	196		
Lysozyme-xanthan gum gels (final pH 7, 25 °C)					14.52 kPa [e], 4 min	[69]
0.3 wt%, ratio 0.5	nd	4.7	4	318		
Electrostatic-induced gels stabilized by heating						
β-lactoglobulin-xanthan gum gels (final pH 4.4, 25 °C → heat treatment 80 °C, 30 min).					14.52 kPa [e], 4 min	[83]
0.36 wt%, ratio 5	nd	2.9	41	164		

nd: not determined
[a] Length scales correspond to the network size at which were observed inhomogeneities from microscopy images [85].
[b] Pore size as determined by ImageJ analysis of confocal micrographs [19]
[c] Values estimated from data presented in each reference.
[d] Centrifugation method as described by Kocher and Foegeding [87].
[e] This value was determined from the equation proposed in [35] and it represents a centrifugation force of 120 g.

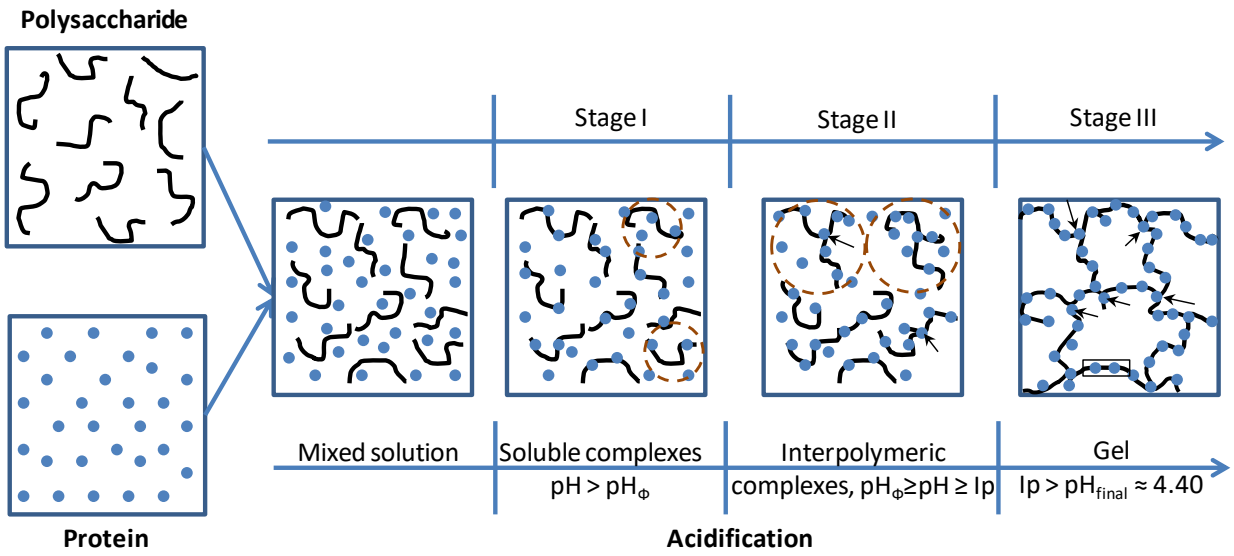

Fig. 1. Mechanism of electrostatic gel formation between β-lactoglobulin and xanthan gum. The arrows represent electrostatic cross-linking zones of xanthan gum chains by β-lactoglobulin and the rectangle highlights the aggregation zone. pHΦ: pH of formation of interpolymeric complexes. Ip: Isoelectric point of the protein.

Adapted from Le and Turgeon [70].

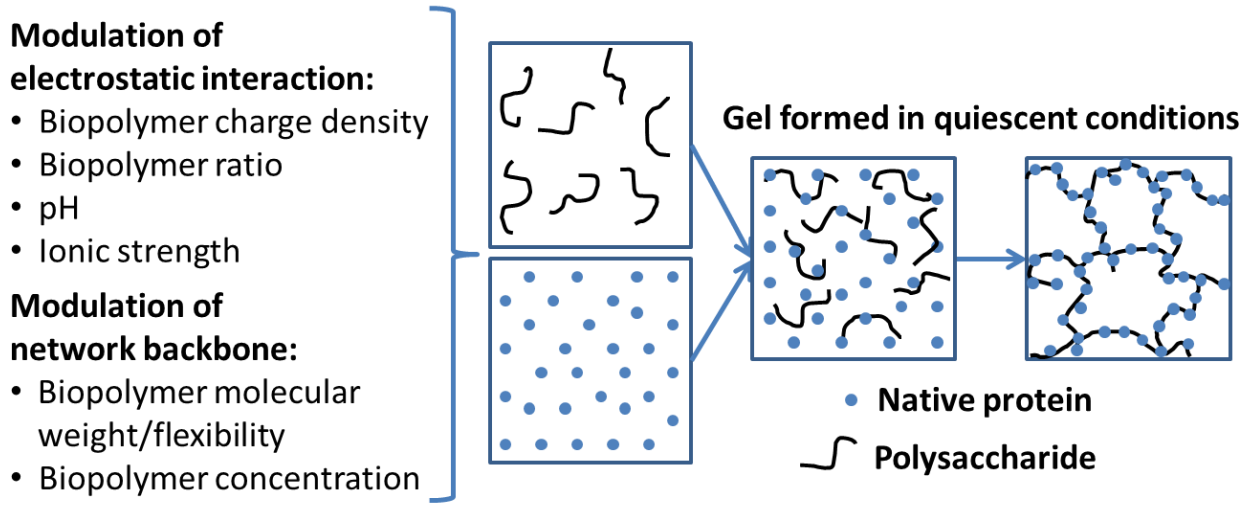

Fig. 2. Factors influencing the formation of electrostatic protein-polysaccharide gels.

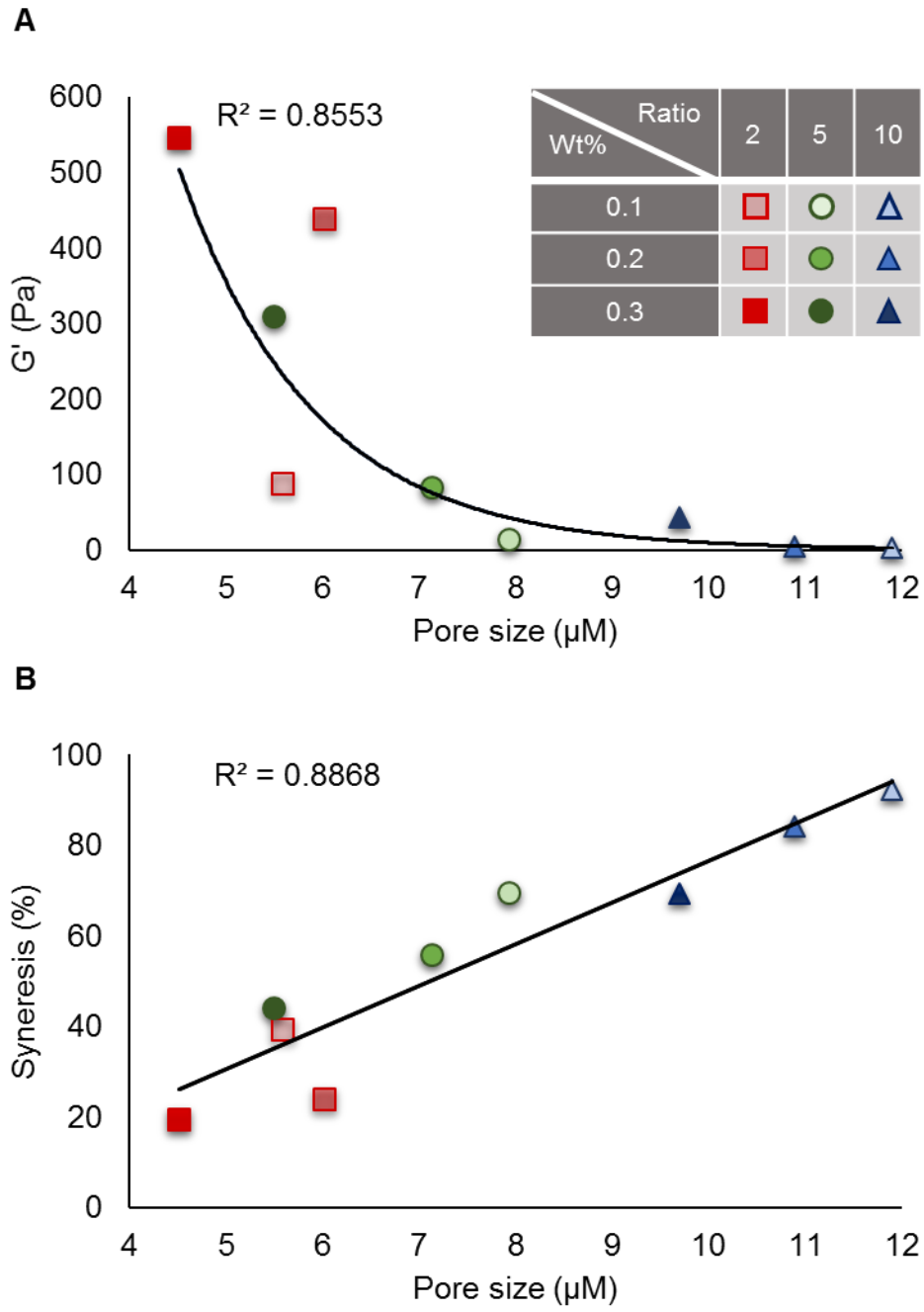

Fig. 3. Relationship between electrostatic gel properties (final G' and syneresis) and the network pore size. The mixtures of β-lactoglobulin and xanthan gum had a total solid concentration of 0.1-0.3 wt% and a ratio of 2, 5 and 10. Adapted from [19].

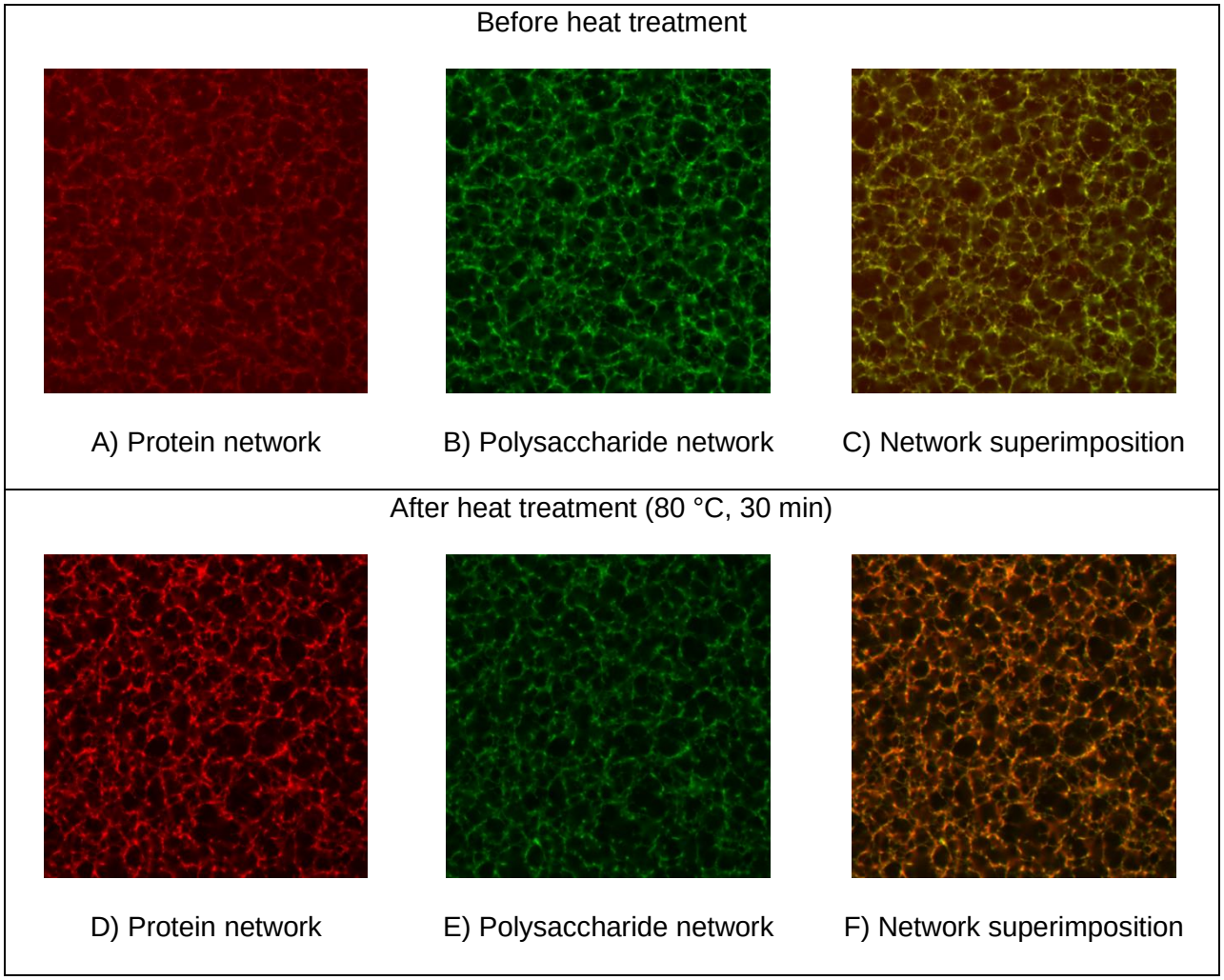

Fig. 4. Effect of heat treatment on of β-lactoglobulin-xanthan gum gel structure observed by confocal laser scanning microscopy. The mixture contained 1.2 wt% of β-lactoglobulin and 0.06 wt% of xanthan gum (ratio 20). The images represent an area of 91.87 x 91.87 µm. Adapted from [83].