**Promotors:** Prof. Dr. Ir. Paul Van der Meeren
Ghent University

Prof. Dr. Zahra Emam-Djomeh
University of Tehran

Prof. Dr. Seyed Hadi Razavi
University of Tehran

**Dean:** Prof. Dr. Ir. Guido Van Huylenbroeck

**Rector:** Prof. Dr. Paul Van Cauwenberge
Ir. Seyed Mohammad Hashem Hosseini

Complex coacervation between β-lactoglobulin and anionic polysaccharides and its potential application for nanoencapsulation of hydrophobic functional components

Thesis submitted in fulfillment of the requirements for the joint degree of Doctor (Ph.D.) in Applied Biological Sciences and Food Science and Technology
Dutch translation of the title:

Complex coacervatie tussen β-lactoglobuline en anionische polysachariden en zijn mogelijke toepassing voor nano-encapsulatie van hydrofobe functionele componenten

To refer to this thesis:


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Mohammad
ABSTRACT

Many beneficial nutraceuticals such as carotenoids, fat-soluble vitamins and phenolic compounds are lipophilic. The hydrophobic nature of these compounds makes their incorporation into non-fat aqueous foods and beverages (especially transparent ones) challenging. The main purpose of this study was the nanoencapsulation of four nutraceutical models in slightly acidic media utilizing the attractive interactions between β-lactoglobulin (BLG) and four anionic polysaccharides. In this study, the intrinsic transporting property of BLG was utilized to develop nano-sized green delivery systems. The binding analysis suggested that BLG-ligand complexation occurred under all conditions but varied as a function of pH and nutraceutical type. The fluorimetry and NMR data provide different but complementary information on BLG-ligand interactions. These findings resulted in designing nanoscopic delivery systems for encapsulation of both hydrophobic and hydrophilic bioactives in liquid and transparent food products of acidic pH. The stability experiments demonstrated that the nutraceuticals of low solubility in water were successfully entrapped within electrostatically stable nanocomplexes arising from protein-polysaccharide interactions. In order to downsize the produced nanoparticles, the interaction between BLG and either sodium alginate (ALG) or κ-carrageenan (KC) (before and after polysaccharide sonication) was also investigated using isothermal titration calorimetry (ITC), streaming current detector (SCD), turbidity measurement, dynamic light scattering and electrophoretic mobility methods. High intensity ultrasound could effectively decrease the viscosity of both polysaccharide solutions. Time and amplitude of the sonication treatment had a direct effect on the viscosity depression, while the sonication temperature had an inverse effect. ITC measurements indicated that the sonication decreased the
interaction strength and/or binding affinity between protein and polysaccharide. The zeta-potential of the nanoparticles produced from sonicated polysaccharide-BLG attractive interaction was lower than of those produced from intact polysaccharide-BLG interaction. These differences were attributed to the lower charge density of the sonicated polysaccharide as a result of sonochemical interactions. Particle size measurements showed that the effect of the sonication treatment was the homogenization of the nanoparticles in the mixed dispersion. Finally, the results of this study showed that the delivery systems formed can be used for fortification purposes of transparent acidic beverages with both hydrophobic and hydrophilic bioactives.

Keywords: Complex coacervation; β-Lactoglobulin; Anionic polysaccharide; Ultrasound; Nanoparticle; Delivery system; Isothermal titration calorimetry; Streaming current detector; Nutraceutical
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<th>Description</th>
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<tbody>
<tr>
<td>1H NMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>ALG</td>
<td>Sodium alginate</td>
</tr>
<tr>
<td>ANS</td>
<td>8-anilinonaphthalene-1-sulfonic acid ammonium salt</td>
</tr>
<tr>
<td>BLG</td>
<td>β-Lactoglobulin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxy methyl cellulose</td>
</tr>
<tr>
<td>CSLM</td>
<td>Confocal scanning laser microscopy</td>
</tr>
<tr>
<td>CUR</td>
<td>Curcumin</td>
</tr>
<tr>
<td>Dₙ</td>
<td>Apparent fractal degree</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DLCA</td>
<td>Diffusion-limited cluster aggregation</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DOSY</td>
<td>Diffusion ordered spectroscopy</td>
</tr>
<tr>
<td>DWS</td>
<td>Diffusing wave spectroscopy</td>
</tr>
<tr>
<td>EEP</td>
<td>Electrical equivalence pH</td>
</tr>
<tr>
<td>EM</td>
<td>Electrophoretic mobility</td>
</tr>
<tr>
<td>FA</td>
<td>Folic acid</td>
</tr>
<tr>
<td>GA</td>
<td>Gum Arabic</td>
</tr>
<tr>
<td>HP</td>
<td>High pressure</td>
</tr>
<tr>
<td>IN</td>
<td>Intact</td>
</tr>
<tr>
<td>Ip</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>IV</td>
<td>Intrinsic viscosity</td>
</tr>
<tr>
<td>K</td>
<td>Affinity constant</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>KC</td>
<td>κ-Carrageenan</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>$N$</td>
<td>Binding stoichiometry</td>
</tr>
<tr>
<td>NATA</td>
<td>$N$-acetyl-$L$-tryptophanamide</td>
</tr>
<tr>
<td>NG</td>
<td>Nucleation and growth</td>
</tr>
<tr>
<td>polyDADMAC</td>
<td>Polydiallyldimethylammonium chloride</td>
</tr>
<tr>
<td>PPM</td>
<td>Parts per million</td>
</tr>
<tr>
<td>SCD</td>
<td>Streaming current detector</td>
</tr>
<tr>
<td>SD</td>
<td>Spinodal decomposition</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasonicated</td>
</tr>
<tr>
<td>VD</td>
<td>Ergocalciferol (vitamin D$_2$)</td>
</tr>
<tr>
<td>ZCP</td>
<td>Zero charge point</td>
</tr>
<tr>
<td>βC</td>
<td>β-carotene</td>
</tr>
<tr>
<td>Δ$C_p$</td>
<td>Heat capacity change</td>
</tr>
<tr>
<td>Δ$G$</td>
<td>Gibbs free energy change</td>
</tr>
<tr>
<td>Δ$H$</td>
<td>Enthalpy change</td>
</tr>
<tr>
<td>Δ$S$</td>
<td>Entropy change</td>
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1. Introduction

1.1. Protein-polysaccharide complexes and coacervates

Proteins and polysaccharides belong to the main components of foods. These two ingredients are firstly fulfilling the nutritional purpose of providing an equilibrated caloric diet to maintain health and well-being for people consuming them (Schmitt, Aberkane, & Sanchez, 2009). They are broadly classified as biopolymers because of their large molecular structures (Goh, Sarkar, & Singh, 2009). These natural biopolymers are used as essential functional ingredients in many technological applications including food, pharmaceutical and biomedical industries, cosmetics, and so forth (McClements, 2006; Corredig, Sharafbafi, & Kristo, 2011; Rodríguez Patino, & Pilosof, 2011). They play a key role in determining the shelf-life, stability, viscoelastic properties and inmouth perception of most food products and also in the formation of the building blocks of structure and texture through rheological control of the aqueous medium caused by aggregation and gelation (Dickinson, 1998; Tolstoguzov, 2003; Dickinson, 2008; Corredig et al., 2011). Polysaccharides are widely used as stabilizers, thickening or gelling agents (Dickinson, 1998; Goh et al., 2009; Corredig et al., 2011) and to form edible films (Hosseini, Razavi, & Mousavi, 2009). Being surface-active, proteins can function as effective emulsifying agents in the formation and stabilization of emulsions and foams; and, being polyelectrolytes, they can impart excellent colloidal stability to emulsion droplets by a combination of electrostatic and steric mechanisms (McClements, 2004; Dickinson, 2008). The long-term stability can be further enhanced using polysaccharides to control the rheology and network structure of the continuous phase, hence retarding phase separation

1
and gravity-induced creaming (Dickinson, 2008). Proteins are also often employed as carriers for other molecules or to protect different active materials, for example, in microencapsulation processes (Ducel, Saulnier, Richard, & Boury, 2005). The physicochemical properties of proteins and polysaccharides have individually been studied extensively over the last several decades. It is well established that the factors influencing the physicochemical properties of these macromolecules in solution include molar mass, molecular conformation, polydispersity, charge density, concentration, pH, ionic strength, temperature, solvent quality and nature of intra- or inter-molecular interactions. The physicochemical properties of the proteins and polysaccharides depend not only on the molecular parameters of the individual biopolymers but also on the nature of interactions between the protein and polysaccharide molecules (Goh et al., 2009).

Tailor-made functionalities such as microencapsulation (McClements, 2006; Jun xia, Hai-yan, & Jian, 2011), nanoencapsulation (Ron, Zimet, Bargarum, & Livney, 2010), interfacial stabilization (Girard, Turgeon, & Paquin, 2002; Schmitt, da Silva, Bovay, Rami-Shojaei, Frossard, Kolodziejczyk, & Leser, 2005; Dickinson, 2008), texturizing such as fat replacing (Laneuville, Paquin, & Turgeon, 2005), formation of novel gels (e.g. electrostatic gels) (van den Berg, van Vliet, van der Linden, van Boekel, & van de Velde, 2007; Picard, Giraudier, & Larreta-Garde, 2009) and development of new functional nano, micro or macrostructures (Benichou, Aserin, & Garti, 2002) can often be introduced into a system by using non-covalent (electrostatic and hydrophobic) interactions as well as hydrogen bonding between biopolymer blends. The new functionalities resulting from these self-assembling structures are of great applied significance for the improvement of many foods and of increasing interest in soft-condensed matter research (de Vries & Cohen Stuart,
2006; Schmitt et al., 2009). A proper understanding and control of these different interactions should enable food scientists to design and fabricate biomacromolecular assemblies in order to develop food products with desired structure and texture (Turgeon, Beaulieu, Schmitt, & Sanchez, 2003; Schmitt et al., 2009).

In an aqueous environment, when proteins which are charged amphoteric molecules come into contact with polysaccharides, one of four phenomena can arise: (a) co-solubility; (b) thermodynamic incompatibility; (c) depletion flocculation; and (d) complex coacervation (Fig. 1.1); mainly depending on the electrical charges on both biopolymers, and therefore on the factors affecting them, such as the pH and the ionic strength as well as on the size and concentration of biopolymer molecules (Ducel, Pouliquen, Richard, & Boury, 2008; Turgeon, & Laneuville, 2009; Goh et al., 2009). These phase behaviors arise from long- or short-range interactions between the biomacromolecules themselves, and possibly because of different affinities between the biomacromolecules and the solvent too (Schmitt et al., 2009). These phenomena which show completely different functional properties will be explained in more detail below.

1.1.1. Co-solubility

Co-solubility, which usually occurs in dilute system, refers to the development of a stable homogeneous solution, i.e. the generation of one phase in which the two macromolecular species either do not interact or exist as soluble complexes in the aqueous medium (as in the case of monomer sugars and hydrophilic amino acids) (Tolstoguzov, 2003; Ye, 2008; Goh et al., 2009). Generally, when intermolecular attraction is inhibited or absent, each macromolecule ignores the presence of the other one. Macromolecular mixtures are co-soluble only in a dilute solution, where
the entropy of mixing favors more randomness in the system (Tolstoguzov, 1997, 2003).

From a thermodynamic viewpoint, the Gibbs’ free energy of mixing ($\Delta G_{\text{mixing}}$), shown in equation (1.1), must be negative to achieve co-solubility.

$$\Delta G_{\text{mixing}} = \Delta H_{\text{mixing}} - T\Delta S_{\text{mixing}}$$  \hspace{1cm} (1-1)

where $\Delta G_{\text{mixing}}$, $\Delta H_{\text{mixing}}$, and $\Delta S_{\text{mixing}}$ are the changes in Gibbs’ free energy, enthalpy and entropy upon mixing, respectively. This means that the entropy of mixing should be greater than the enthalpy of mixing (Goh et al., 2009). The highest level of entropy is accomplished when the different kinds of molecules are randomly distributed throughout the system (McClements, 2005). In other words, for the free energy of mixing of small molecules, the unfavorable thermodynamic contribution
from a relatively modest endothermic term is usually overwhelmed by the entropic ideal mixing term. This then favors miscibility unless the liquid mixture is extremely non-ideal (Dickinson, 1998). When biopolymer size (molecular weight) and concentration increase and exceed a certain critical value, the biopolymers become limitedly co-soluble caused by the bulky size and rigidity of the molecules resulting in a decrease in entropy of mixing (or higher free energy or thermodynamic incompatibility) (Tolstoguzov, 1997, 2003). Therefore, the entropy of mixing of biopolymers is significantly lower than that of the monomers. The low biopolymeric entropy of mixing means that, if the protein-polysaccharide interaction is only slightly net repulsive, the system prefers to exist as two separate phases when the overall biopolymer concentration reaches just a few per cent (Dickinson, 1998). In spite of a co-solubility of monomer sugars and hydrophilic amino acids, proteins and polysaccharides are normally limitedly compatible, i.e. limitedly miscible on a molecular level (Tolstoguzov, 2003).

For a mixed biopolymer solution having a net repulsive interaction, the enthalpy–entropy balance generally results in mutual exclusion of each biopolymer from the local vicinity of the other (Dickinson, 1998; Goh et al., 2009). This means that macromolecules in mixed solution show a preference to be surrounded by their own type. Consequently, their mixtures separate into liquid phases (Tolstoguzov, 1997, 2003; Goh et al., 2009). Normally, the excluded volume of macromolecules determines their phase separation conditions (Tolstoguzov, 2003). Excluded volume or steric exclusion is a short-range repulsive interaction arising from strongly unfavorable overlap of electron clouds (Dickinson, 1998). The thermodynamic driving force for this type of interaction is a configurational entropy effect (i.e. a change in the number of configurations available to the molecules or particles in the system)
Excluded volume effect is important for food components that are capable of occupying relatively large volumes within a system, thereby excluding other components from occupying the same volume (McClements et al., 2009). Indeed steric interactions restrict the relative spatial arrangement of pairs of segments on the same or different macromolecules (Dickinson, 1998). Fig. 1.2 shows the concept of excluded volume for globular protein and linear rigid polysaccharide molecules (Tolstoguzov, 2003).

**Fig. 1.2:** The concept of excluded volume (EV).

It shows two neighboring impenetrable spherical molecules of the same radius (R). Consequently, a minimal distance between two protein molecules equals the sum of their radii or the diameter (D) of one of them. This means that the radius of the excluded volume around each protein molecule equals the diameter of the macromolecule. In other words, the excluded volume, from which the centers of other protein molecules are ejected, is eight-fold greater than that of the molecule itself. Excluded volume is significantly greater for non-spherical macromolecules, e.g. linear rigid polysaccharides. In dilute solution, stiff rod-like macromolecules are
relatively independent when the distance between them equals or is larger than their length. Excluded volume effects reflect mutual competition between macromolecules for solution space. Excluded volume effects tend to become more important as the effective volume of the molecules or particles increases and as their molar concentration in the system increases (McClements et al., 2009). A decrease in the excluded volume with increasing concentration of macromolecules results in small repulsive interactions or phase separation (Tolstoguzov, 2003).

1.1.2. Thermodynamic incompatibility

Thermodynamic incompatibility also known as segregative phase separation, occurs at high concentrations and high ionic strengths, when the two non-interacting macromolecular species mutually segregate into two different distinct immiscible aqueous phases, one phase mainly rich in one biopolymer (e.g. protein) and the other phase mainly rich in the other biopolymer (e.g. polysaccharide) (Dickinson, 1998; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003; Ye, 2008; Turgeon et al., 2009; Goh et al., 2009). In this case, biopolymers have net repulsive interactions which are non-specific and typically of transient duration. They mainly arise from excluded volume effects and ionic forces between like charges, and they tend to be weak except at close range (hard-sphere interactions) or very low ionic strength (unscreened electrostatic interactions) (Dickinson, 1998). From a thermodynamic viewpoint, the enthalpy of mixing exceeds the entropy difference (Tolstoguzov, 2002; Goh et al., 2009). In thermodynamic incompatibility, each biopolymer shows varying affinity towards the solvent. Solvent–protein (or solvent–polysaccharide) interactions are favored over protein–polysaccharide interactions and solvent–solvent interactions (Doublier, Garnier, Renard, & Sanchez, 2000). Thermodynamic incompatibility can also arise within a mixture of polysaccharides or proteins. Some
examples include: polysaccharides with different structures; proteins of different classes, such as water-soluble albumins with salt-soluble globulins; native and denatured forms of the same protein as well as aggregated and non-aggregated forms of the same protein (Tolstoguzov, 2002). Thermodynamic incompatibility is highly dependent on pH, ionic strength and overall biopolymer concentration and is prevalent when protein and neutral polysaccharide are present or when both protein and polysaccharide carry the same electrical charge (Doublier et al., 2000).

A parameter which is related to the nature of the interaction between protein (PR) and polysaccharide (PS) in dilute solution is the cross second (A₂) virial coefficient (A_{PR+PS}) determined in a static light-scattering experiment (Dickinson, 1998). Generally, virial coefficients are the quantitative measure of non-specific intermolecular interactions as mediated by the solvent; their variation upon changing the buffer reveals how the interactions between molecules are affected by the solvent. Virial coefficient analysis can be used both for self-interactions, that is interactions between like molecules and cross interactions, namely interactions between different types of molecules. Cross virial coefficients are measured by taking two types of molecules, varying their relative concentrations and measuring the scattered light. At each step, the total light scattering signal is divided by the total concentration to obtain the apparent molar mass. The slope of apparent molar mass vs. concentration yields the second virial coefficient (A₂) (Some, Hitchner, & Ferullo, 2009). Negative and positive values of A_{PR+PS} are indicative of net attractive and net repulsive interactions, respectively (Dickinson, 1998). In the absence of multibody effects, the thermodynamic behavior of a protein-polysaccharide solution depends on the relative values of A_{PR+PS}, A_{PR+PR} and A_{PS+PS}, where the quantities A_{PR+PR} and A_{PS+PS} are pure protein and pure polysaccharide virial coefficient, representing the
thermodynamic contributions from protein-protein and polysaccharide-polysaccharide interactions, respectively (Dickinson, 1998). Values of $A_{PR+PS}$ for many non-dilute binary protein/polysaccharide mixtures are such that thermodynamic incompatibility is prevalent (Dickinson, 1998; Goh et al. 2009), however, some of these systems do not achieve thermodynamic equilibrium within a limited timescale because of the presence of kinetic energy barriers. When the kinetic energy exceeds the thermal energy of the system, the molecules become trapped in a metastable state (McClements, 2005). Some examples of kinetic energy barriers include the formation of a gel network within an incompatible system or a highly viscous continuous phase that slows down the phase separation process. The choice of which phase to gel and the component used to promote gelation depends on the type of biopolymers used in the system (Goh et al., 2009).

1.1.3. Depletion flocculation

Depletion flocculation (interaction) refers to the phase separation of small spheres (proteins or oil droplets) in the presence of macromolecules, which is enhanced by the addition of coils (polysaccharides). This phenomenon usually occurs in a colloidal dispersion in the presence of non-interacting biopolymers (e.g. polysaccharides in an emulsion, polysaccharides and colloidal casein micelles) (Goh et al., 2009). The higher osmotic pressure of the polymer molecules surrounding the colloidal particles (as compared to the inter-particle region) causes an additional attractive force between the particles, leading to their flocculation (Goh et al., 2009). The attractive force depends on the size, shape and concentration of the polymer molecules and the colloidal particles. When colloidal particles approach each other, the excluded (or depleted) layer starts to overlap, leading to formation of spherical protein-rich domains that precipitated slowly and hence allowing polymer molecules
to occupy more space (Turgeon et al., 2003; Goh et al., 2009). The increase in volume causes the total entropy of the system to increase (i.e. the free energy to decrease), which in turn encourages attraction interaction between the colloidal particles. In a binary protein-polysaccharide system, where the protein species is casein micelles, phase separation is often attributed to a depletion flocculation phenomenon, because of the large colloidal particle size of the casein micelles and because increasing the concentration of polysaccharides results in greater attraction between the casein micelles (Doublier et al., 2000).

1.1.4. Complex coacervation

Complex coacervation also known as thermodynamic compatibility or associative phase separation usually occurs at relatively lower concentrations (< 3-4% w/w total biopolymer concentration) as compared to thermodynamic incompatibility, and also at low ionic strengths (< 300 mM), and when proteins and polysaccharides exert strong attractive interactions (Tolstoguzov, 2007; Ye, 2008; Schmitt et al., 2009; Turgeon et al., 2009). In good solvent conditions, complex coacervation occurs at a pH between the proteins’ isoelectric point (Ip) and the pKₐ of the polysaccharide (Tolstoguzov, 1997; de Kruijf, Weinbreck, & de Vries, 2004). Under such conditions, biopolymers spontaneously associate by excluding solvent (water) from their vicinity and form primary soluble macromolecular complexes (Doublier et al., 2000; Turgeon et al., 2003). These complexes generally further aggregate forming a three-dimensional network due to charge neutralization. Because the complexes remain highly hydrated, they form a dispersion of liquid droplets concentrated in biopolymers (so-called coacervates) that tend to coalesce in order to minimize their interfacial free energy, leading to the formation of two separate phases (on a macroscopic level), the lower phase (so-called coacervate
phase) rich in both protein and polysaccharide and the upper one depleted in biopolymers but rich in solvent (so-called equilibrium solution) (Dickinson, 1998; Ducel et al., 2004; de Kruif et al., 2004; Schmitt et al., 2009; Goh et al., 2009; Turgeon et al., 2003, 2009). The protein and polysaccharide in the coacervate phase are held together mainly through electrostatic interactions (when they have oppositely charged groups) and can take the form of a dense liquid coacervate phase or a solid precipitate (Doublier et al., 2000; Ye, 2008; Turgeon et al., 2009; Schmitt et al., 2009). If the two biopolymers are present in equal proportions by weight at a pH such that they carry net equal opposite charges, the yield of coacervate will be at its maximum (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). It is important to note that complex coacervation may occur by bad (poor) solvent conditions in which a concentrated phase can be obtained without requiring the involvement of attractive interactions between biopolymers (Doublier et al., 2000; Schmitt et al., 2009).

1.2. Phase diagram

A typical phase diagram for a protein–polysaccharide solution with water as the solvent at a particular pH, temperature and ionic strength is shown in Fig. 1.3 and has been explained by Goh et al. (2009). The phase diagram consists of a typical binodal curve (the solid line curve), which separates the single-phase miscible domain underlying the binodal curve from the two-phase immiscible domain lying above the binodal curve, which is obtained by direct observation of the phase separation in test tubes. The binodal branches which do not coincide with the phase diagram axes correspond to a limited co-solubility of the biopolymers. They are water-in-water (W/W) emulsions. A W/W emulsion is a disperse system, where
droplets of one of the immiscible aqueous solutions are dispersed throughout another aqueous biopolymer solution (Tolstoguzov, 2003).

**Fig. 1.3:** A typical phase diagram showing a protein–polysaccharide solution with water as the solvent at a particular pH, temperature and ionic strength, reproduced from Goh et al. (2009).

The points of the binodal curve connected by tie lines represent the compositions of the co-existing equilibrium phases. From the phase diagram, it is possible to determine the effective concentrations of biopolymers in the two phases and the concentrations at which maximal co-solubility of the biopolymers is achieved. In addition, it helps to establish which of the two biopolymers forms the continuous phase. For example, a sample of composition O (which was initially made with A% protein and B% polysaccharide) separates out into two bulk polymer-rich phases. The protein-enriched phase will have composition C% protein whereas the polysaccharide-enriched phase will have composition D% polysaccharide. The
protein content in the polysaccharide phase will be negligible and vice versa. The tie line is obtained by joining C and D. The ratio DO/OC represents the volume ratio of the protein-rich phase C and the polysaccharide-rich phase D estimated by the inverse-lever rule. When the composition of the system O is shifted along the tie-line to O₁, the volume ratio of the co-existing phases is changed, but their compositions (effective concentration in the enriched phases) remain constant. The line obtained by joining the mid points (+) of two or more tie lines gives the rectilinear diameter. The co-ordinates of the critical point E (obtained from the intersection of the binodal curve to the rectilinear diameter) gives the composition of systems splitting into two phases of the same volume, which means that the separated-phase systems will have 50% protein and 50% polysaccharide in the same phase volume ratio. Point F is the phase separation threshold, which is the minimum critical concentration of biopolymers required for phase separation to occur. Normally, in terms of biopolymer incompatibility, foods are phase separated and highly volume occupied systems. This means that the concentration of the biopolymers in a food usually exceeds their phase separation threshold (Tolstoguzov, 2003). The phase diagram shows that minor changes in food formulation, especially near the critical point and near the rectilinear diameter, can change the composition of the continuous phase, and consequently the texture, flavor and other qualities of the food. This illustrates the fact that the functionality of an added biopolymer will greatly depend upon food formulation (Tolstoguzov, 2003).

1.3. Development of the water-in-water (W/W) emulsions

Phase separating protein–polysaccharide systems can be considered as a W/W emulsion with some particularities including the co-solubility of the biopolymers
in the coexisting phases in an equilibrium state and the low density and viscosity of the interfacial or depletion layer between immiscible aqueous phases resulting in low values \(10^{-6} \text{ N m}^{-1}\) of interfacial tension (Turgeon et al., 2003; Tolstoguzov, 2003) as a result of the similar composition of the two coexisting phases. The depletion layer is significant rheologically and its thickness exceeds the macromolecular size, since it is formed to diminish unfavorable interactions between dissimilar macromolecules. The interfacial layer can adsorb hydrophobic particles such as cells and lipids (Tolstoguzov, 2003). The two other features of W/W emulsions, which are of importance for mixing of formulated food components, are the high deformability of dispersed particles and their coalescence in a flowing W/W emulsion which are due to the low interfacial tension, the similar viscosity of the coexisting phases and the low viscosity of the interfacial layer (Tolstoguzov, 2003). Dispersed particles can be easily deformed in flow when their viscosity is lower than that of the continuous phase. Spherical dispersed droplets can greatly deform, orient, coalesce and form long liquid filaments. The latter are not stable and break up into smaller drops. Deformation of a W/W emulsion followed by gelation of its phases, which prevents demixing, results in the generation of particles with specific shape and size (e.g. anisotropic materials of fibrous or lamellar structure (Tolstoguzov, 2003).

1.4. Nature of the interactions in associative protein–polysaccharide systems

Knowledge of the origin and nature of the various molecular and colloidal forces that act between food components is essential for understanding how to rationally assemble structured systems with specific functional performances (McClements et al., 2009). Attractive interactions vary widely in strength and specificity. The overall interaction between protein and polysaccharide is made up from an average over the
large number of different intermolecular forces arising between the various segments and side-chains of the two biomacromolecules (Dickinson, 1998; Schmitt et al., 1998; Goh et al., 2009). A brief discussion of the major interactions responsible for the formation of the supramolecular structures is given in this section.

1.4.1. Covalent bonding

Covalent bonding is obviously a highly specific, very strong and non-electrostatic linkage formed between specific reactive groups on different macromolecules which confers permanence to protein-polysaccharide complexes (Dickinson, 1998; Goh et al., 2009). Covalent bonding between proteins and polysaccharides can be generated using the Maillard reaction (chemical reaction between amino groups of proteins and carboxylic groups of polysaccharides to give an amide covalent bond) and/or enzymatically using the oxidoreductase family of enzymes (Goh et al., 2009). This enzyme family catalyzes the oxidation of the phenolic group of tyrosine residues with carbohydrate groups containing phenolic residues, such as cereal arabinoxylans (Boeriu et al., 2004). Another method which can be utilized to form a covalent linkage between protein and polysaccharide is the cross-linking using transglutaminase (Gan, Cheng, & Mat Easa, 2008; Heidebach, Först, & Kulozik, 2009). Since covalent linkages are generally very stable to changes in pH and ionic strength, this type of interaction can be used to produce conjugated emulsifiers (Neirynck, Van der Meeren, Bayarri Gorbe, Dierckx, & Dewettinck, 2004).

1.4.2. Electrostatic (coulombic) interactions

Electrostatic (coulombic) interactions are the most important forces involved in complex formation between food components that have an electrical charge under the conditions where they are used, e.g., proteins, ionic polysaccharides, ionic surfactants, phospholipids, mineral ions, acids, and bases (Dickinson, 1998; Goh et
Many food components have weak acid groups (e.g., \(-\text{COOH}\)) or base groups (e.g., \(-\text{NH}_2\)) and so their electrical charge is dependent on the prevailing pH relative to the pK\(_a\) of the ionizable groups (McClements et al., 2009). Electrostatic interactions may be either attractive or repulsive depending on whether the charged groups involved have opposite or similar signs (Dickinson, 1998). Electrostatic attraction is commonly used as the driving force to assemble charged food components into specific structures, e.g., multilayers or coacervates (McClements et al., 2009). The electrostatic interaction, which occurs when two or more charged groups come into close proximity, leads to a decrease in the electrostatic free energy of the system. This interaction is comprised of an enthalpy component due to changes in the overall electrical forces (or interactions of oppositely charged biopolymers) and an entropy component due to liberation of counter-ions along with water molecules which compensates for the loss of configurational entropy of mixing rigid biopolymers (Tolstoguzov, 1997; Goh et al., 2009; McClements et al., 2009). Since the overall electrostatic interactions between food components are sensitive to variations in pH and ionic strength, the most common means of manipulating these kinds of interactions is therefore to alter the pH and/or ionic strength of the aqueous solution (Dickinson, 1998; McClements et al., 2009). The strength and range of electrostatic interactions decreases with increasing ionic strength due to electrostatic screening effects (McClements et al., 2009). A specific type of electrostatic interaction that is commonly used in the assembly for food components is *ion bridging*, involving the binding of polyvalent cations (especially Ca\(^{2+}\)) to two different anionic groups (Dickinson, 1998; Goh et al., 2009; McClements et al., 2009).
1.4.3. Hydrophobic Interactions

Hydrophobic Interactions which are moderately strong (5-10 kJ/mol) long-range attractive interactions arise between food components that have non-polar groups when they are dispersed in aqueous solutions, and they manifest themselves as a tendency for the non-polar groups to associate with each other (Dickinson, 1998; McClements et al., 2009; Goh et al., 2009). The molecular origin of the hydrophobic interaction is the fact that water molecules can form relatively strong hydrogen bonds with other water molecules, but not with non-polar groups. The hydrophobic interaction is comprised of an enthalpy component due to the change in the overall strength of the forces (e.g., hydrogen bonding and Van der Waals forces) when two or more non-polar groups associate, and an entropy component due to the change in the structural organization of the water molecules surrounding the non-polar groups (McClements et al., 2009). Hydrophobic bonding is promoted by conformational and structural modifications of biopolymers, mostly by unfolding of polymeric chains exposing hydrophobic groups (Goh et al., 2009). Hydrophobic interactions tend to increase in strength when the temperature is increased (up to ~60 °C.), and decrease in strength when the dielectric constant of the aqueous phase is decreased (e.g., by adding alcohol) (Dickinson, 1998; McClements et al., 2009).

1.4.4. Hydrogen bonding

Hydrogen bonding, a moderately strong (10-40 kJ/mol) short-range attractive interaction with a specific orientational character, is important for food components that have polar groups that are capable of forming hydrogen bonds with other polar groups on the same or on different molecules (Dickinson, 1998; McClements et al., 2009). These bonds are ionic in nature and are formed between a lone pair of electrons on an electronegative atom (such as oxygen and sulfur), and a hydrogen
atom attached to an electronegative atom on a neighboring group (Goh et al., 2009; McClements et al., 2009). Hydrogen bonds tend to decrease in strength as the temperature is increased. Hydrogen bonding is partly responsible for the molecular structures found within many types of food biopolymer (e.g., helical or sheet-like regions) and for holding molecules together in various aggregates and gels (e.g., in gelatin gels) (McClements et al., 2009). Protein–polysaccharide hydrogen bonding has been well established in the complex coacervation of gelatin and pectin, which is obtained over a wide range of pH including the isoelectric point (4.8) of gelatin (Braudo, & Antonov, 1993). Hydrogen bonding and hydrophobic interactions are actually collective interactions (e.g. electrostatic, Van der Waals’ and steric overlap) including some entropy effects (McClements, 2005).

1.4.5. Van der Waals forces

Van der Waals forces are universal weakly attractive interactions of electromagnetic origin exhibited by groups with permanent (dipole-dipole interaction) or induced (London forces) dipoles (Dickinson, 1998; Goh et al., 2009). The permanent and/or temporary dipole in one atom can induce a corresponding dipole in another atom. This is possible only if the atoms are close (Goh et al., 2009). So, Van der Waals forces act between all groups to some extent (Dickinson, 1998). However, if they are too close, repulsive forces between the adjacent negatively charged electron clouds may not allow these Van der Waals attractions (Goh et al., 2009). Although these transient electrical attractive forces are very weak, they influence macromolecular interactions (Goh et al., 2009).

The relative importance of the above mentioned interactions in a particular system depends on the types of food components involved (e.g. molecular weight, charge density vs. pH profile, flexibility, hydrophobicity), the solution composition
(e.g., pH, ionic strength, and dielectric constant) and the environmental conditions, (e.g. temperature, shearing). By modulating these parameters it is possible to control the interactions between the food components and therefore assemble novel structures (McClements et al., 2009).

1.5. Multi-scale structure of protein–polysaccharide complexes and coacervates

Depending on the above-mentioned factors, protein-polysaccharide electrostatic association may result in the formation of different types of structures including coacervates (liquid in nature), complexes (either soluble or insoluble, the latter are also known as interpolymeric complexes or amorphous co-precipitates) and gels (network stabilized by electrostatic interactions) (Dickinson, 1998; Turgeon et al., 2009). The determination of the structure, at the molecular, meso- and macroscopic levels, of protein-polysaccharide associative phase separation represents one of the more challenging and exciting facets of such demixing phenomena (Doublier et al., 2000). Structurally, coacervates separate as dense and structured spherical droplets (vesicles), interpolymeric complexes exhibit fractal aggregates and the gel is composed of an interconnected network of complexes (Ducel et al., 2008; Turgeon et al., 2009). Coacervates are liquid in nature and remain in a liquid state. Since coacervate droplets rich in polymers are dispersed in the continuous phase, systems that form coacervates have sometimes been referred to as liquid/liquid (w/w) emulsions (Ducel et al., 2008; Turgeon et al., 2009). At higher protein/polysaccharide ratio, the stabilization provided by the polysaccharide is no longer efficient and coarsening follows rapidly. In other words, the interaction between coacervates can lead to coalescence and formation of transient
multivesicular structures that will coalesce further and eventually completely sediment into a dense coacervated phase (Sanchez, Mekhloufi, Schmitt, Renard, Robert, Lehr, Lamprecht, & Hardy, 2002). Irrespective of the systems under study, the coarsening processes leading to the formation of a coacervated phase and especially their kinetics are poorly known (Doublier et al., 2000). Coacervates have great interfacial properties due to the adsorption of droplets on the surface. Otherwise, coacervate droplets can be stabilized and utilized for ingredient encapsulation, taste masking and controlled release purposes due to their ability to form a protective film (Ducel et al., 2008; Turgeon et al., 2009).

The internal structure of β-lactoglobulin-acacia gum coacervates exhibits vesicular to sponge-like properties with numerous spherical inclusions (vacuoles) of water (Fig. 1.4) (Schmitt, Sanchez, Lamprecht, Renard, Lehr, de Kruif, & Hardy, 2001).

![Fig. 1.4: Confocal scanning laser micrograph of aggregate free beta-lactoglobulin/gum Arabic coacervate (ratio 1:1) at pH 4.2 and 1% w/w total biopolymer concentration after 3–5 min of production using pre-blending acidification method, reproduced from Schmitt et al. (2001).](image)

Sanchez et al. (2002) explained the origin of the vesicular structure in β-lactoglobulin-acacia gum coacervates by the interfacial presence of acacia gum that
was not electrostatically neutralizing β-lactoglobulin, so that water was entrapped. Additionally, the structure of coacervates as bi- or multi-layers exhibiting a hydrophilic-hydrophilic polarity could also lead to the entrapment of the water molecules (Turgeon et al., 2003). This structure can also be attributed to the presence of non-electrostatic (local) interactions of the layers to give a sponge-like structure (Schmitt et al., 2001; Sanchez et al., 2002). Upon time flow, neutralization proceeds, leading to rearrangements within the coacervates and disappearance of the vacuoles (Turgeon et al., 2003). The diffusivity of whey protein and gum Arabic within their liquid coacervated phase was decreased as compared to that of the original dilute biopolymer mixtures (Weinbreck, Rollema, Tromp, & de Kruif, 2004), because of the higher biopolymer concentration and also due to the electrostatic interactions. Based on the results of fluorescence recovery after photobleaching of the coacervate phase, Weinbreck et al. (2004) showed that whey protein molecules diffuse ten times faster than gum Arabic molecules indicating an independent diffusion. These results suggest that the structure is in continuous movement and the proteins change their binding location on the polysaccharide chains very rapidly (Turgeon et al., 2009). The model which can elucidate this behavior is a mesophase model. In this model, proteins exist in separate microdomains, one domain being more concentrated in proteins and polyelectrolytes (Kayitmazer et al., 2007) (Fig. 1.5). Furthermore, cryo-TEM showed some protein rich zones (Turgeon et al., 2007). However, the diffusivity of the protein greatly decreases around the optimal pH of interaction (Kayitmazer et al., 2007). The unique feature of the coacervate is the presence of extensive dilute domains in which partially interconnected dense domains (50–700 nm) are embedded (Turgeon et al., 2007). Confocal scanning laser microscopy (CSLM) showed that both the protein and the polysaccharide are found
at the interface of the coacervates (Schmitt et al., 2001). Finally, the coacervated phase composition can change with time (Weinbreck et al., 2004a), which can have a significant effect on the coacervate’s functional properties (Turgeon et al., 2007).

**Fig. 1.5:** (a) Phase contrast micrograph of protein - polysaccharide coacervates; (b) a schematic representation of its internal structure reproduced from Turgeon et al. (2009).

Complexes are protein-polysaccharide aggregates of fractal nature which may remain soluble or not, depending on the overall charge they bear (Turgeon et al., 2009) (Fig. 1.6 and 1.7). Soluble complexes are obtained when the opposite charges carried by the two biopolymers are not equal in number (i.e. at high polysaccharide
to protein ratios and at moderate ionic strengths or when the biopolymers have low charge densities or when the pH of the system is relatively far from the isoelectric point of the protein), whereas insoluble complexes result when the net charge on the complex is close to zero (Goh et al., 2009; Turgeon et al., 2009).

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**Fig. 1.6:** Phase contrast micrograph of beta-lactoglobulin – sodium alginate interpolymeric complexes

**Fig. 1.7:** Scanning electron micrograph of beta-lactoglobulin – gum Arabic soluble complexes

Soluble complexes consist of single polysaccharide chains bearing only a few protein molecules. Such complexes still carry enough overall negative charge to remain soluble (Turgeon et al., 2009) due to the electrostatic repulsion. If more
protein molecules bind to the polysaccharide chain, charge neutralization is obtained resulting in the formation of interpolymeric (insoluble) complexes (Turgeon et al., 2009). In order to follow the evolution of the internal structure of the emerging complexes, apparent fractal degree (d_f) measurement using light-scattering techniques is the method of choice (Girard, Sanchez, Laneuville, Turgeon, & Gauthier, 2004). The d_f value shows the compactness degree of a structure: if particles aggregate in linear arrays the d_f value tends to 1; whereas if they form compact spherical aggregates, the d_f tends to 3 (Turgeon et al., 2009). Soluble complexes exhibit different internal structures. They can be denser or looser as compared to their respective interpolymeric complexes (Girard et al., 2004). Soluble and interpolymeric complexes have excellent texturing and stabilizing properties. Additionally, soluble complexes can also have good interfacial properties (Turgeon et al., 2009). The internal structure of insoluble complexes is much denser and the molecules in them are less motile as compared to a coacervated phase (Turgeon et al., 2009). This compactness has its roots in thermodynamics. If the polyelectrolyte has a stiff conformation, the configurational entropy loss upon complexation will be larger than if it was a random coil which may facilitate interaction. On the other hand, stiff polyelectrolytes will try to retain their original conformation to minimize entropy loss (Turgeon et al., 2009). As a result, the protein will tend to occupy the interstitial spaces of the polyelectrolyte chain resulting in a denser structure (Li, Xia, & Dubin, 1994). During the coarsening process to achieve the final equilibrium structure, molecular rearrangements can occur. The time needed to reach equilibrium (the formation of neutralized complexes) is dependent on the mixing conditions and the protein content of the system, ranging between some hours and some days (Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2003).
An electrostatic gel can be obtained from the associative interaction between proteins and polysaccharides. The three-dimensional structure of this type of gel is stabilized primarily by attractive electrostatic interactions. The most important features of this gel as compared to other protein gels are that it can be formed at extremely low (< 0.5% w/w) concentrations, and that the protein in the system is in a native (intact) state, since it has not been subjected to any denaturing process (thermal, enzymatic, etc.). The gelation process occurs under specific conditions (slow acidification, quiescent conditions) and follows, at least initially, a similar path as that of complexation, i.e. with the formation of primary soluble complexes followed by interpolymeric electrostatic complexation. However, interpolymeric complexes are able to form junction zones that result in the freezing of the whole structure and at the point of gelation coarsening and phase separation (complexes precipitation) are completely inhibited (Turgeon et al., 2009). The internal structure and firmness of the electrostatic gels can be modulated by choosing proteins and polysaccharides of different origin or by adjusting other parameters like mixing ratio, ionic strength and total biopolymer concentration. This type of gel has potential application as a texturizing agent or as a delivery system (Turgeon et al., 2009).

1.6. Coacervation or complexation?

The fundamental question here is based on which factors protein-polysaccharide mixed systems lead to either coacervation or complexation?

Generally, it seems that the factors affecting interpolymeric complexes and/or coacervates formation are unique to each protein-polyelectrolyte mixed system and depend on the specific characteristics of the biomacromolecules in the mixture; e.g. low-methoxyl-pectin will form coacervates with gelatin (a low-charge-density
polyampholyte), whereas it will form interpolymeric complexes with β-lactoglobulin (Turgeon et al., 2009). Although coacervate and complex developments follow the same initial path, it is still not clearly understood why some protein-polysaccharide mixed systems phase separate as interpolymeric complexes whereas others result in coacervates (Turgeon et al., 2009). Generally, the formation of interpolymeric complexes versus coacervates could be related to the stiffness and to the charge density of polysaccharides and proteins (Turgeon et al., 2007). Highly charged and more stiff biopolymers (e.g. κ- and ι-carrageenan, sodium alginate, gellan, or xanthan gum) lead to interpolymeric complexes (aggregates), while low (intermediate) charge density and flexible ones (e.g. gelatin, acacia gum, dextran sulfate, sodium hyaluronate and some specific species of pectin) lead to coacervates (Turgeon et al., 2007, 2009). The formation of interpolymeric complexes may also be related to the interaction strength (binding affinity). When the binding affinity is high, proteins bind tightly to the polysaccharide backbone inducing extensive counterion release (Turgeon et al., 2009). This entropically driven effect induces desolvation to the point that co-precipitation is preferentially obtained instead of coacervation (Kayitmazer, Strand, Tribet, Jaeger, & Dubin, 2007b). It seems that in some systems, hydrogen bonding or hydrophobic interactions may also have an important role in the formation of coacervates versus interpolymeric complexes (Nigen, Croguennec, Renard, & Bouhllab, 2007; Kumar, Dubin, Hernon, Li, & Jaeger, 2007). Kumar et al. (2007) reported that the molecular weight (MW) of the polyelectrolyte can play an important role. Larger MW is necessary for the coacervate formation and lower MW induces interpolymeric complexes. The latter was attributed to the greater impact of chain configurational entropy loss upon complexation resulting in a larger decrease in the free energy of the system (Kumar
et al., 2007). The sensitivity of coacervation on the macromolecular structure was evidenced upon addition of only one carbon atom in the zwitterionic Gemini surfactant chain, leading to either a transparent gel or a coacervate at a concentration of 5% (w/w) (Menger, Seredyuk, Apkarian, & Wright, 2002).

1.7. Conformational changes as affected by protein-polysaccharide complexes

On a molecular level, complex formation can cause molecular changes to both the protein and the polysaccharide components. It was shown that β-casein reduced the helical structure of carrageenans upon complexation with them and the resulting complexes were soluble over a wider pH range than β-casein (Burova, Grinberg, Grinberg, Usov, Tolstoguzov, & de Kruif, 2007). This was an indication that the electrostatic interactions between the two biopolymers were preventing the formation of the secondary hydrogen bonds that are responsible for the helical structure of the bare polysaccharide (Schmitt et al., 2009). It has also been reported that complexation can cause the loss of micellization-ability in proteins such as β-casein (Burova et al., 2007). An interesting finding is that molecular changes of proteins can occur before or after complexation. In the former case, changes in the protein conformational structures can favor the interaction with polysaccharides and are sometimes necessary for it (Turgeon et al., 2007). It was also found that complex coacervation between β-lactoglobulin and acacia gum induces a loss (about 50%) in the amount of α-helix protein structure after complexation as determined by circular dichroism and apparent ellipticity measurements (Schmitt, Sanchez, Despond, Renard, Robert, & Hardy, 2001; Mekhloufi, Sanchez, Renard, Guillemin, & Hardy, 2005). The most likely explanation for this observation was that this region of the protein was rich in positively charged basic amino acids probably strongly interacting
with the carboxylic groups from the acacia gum. These amino acid residues were also exposed to the solvent at the surface of the protein (Schmitt et al., 2009, 2011). After the bulk phase separation, protein regained secondary structure suggesting a molecular reorganization probably induced by protein concentration in the coacervate (Mekhloufi et al., 2005). Nevertheless, depending on the structure of the protein, complex formation may also lead to an increase in $\alpha$-helix as, for example, upon complex formation between poly(L-lysine) and $\iota$-carrageenan (Girod, Boissière, Longchambon, Begu, Tourne-Pétheil, & Devoisselle, 2004). Protein conformational changes can even occur between a protein and a neutral polysaccharide, as reported between bovine serum albumin (BSA) and high molecular weight dextran in water (Antonov, & Wolf, 2005). Another interesting finding is that the protein denaturation temperature was directly linked to the number of reactive sites of the protein able to interact with the polysaccharide. Thus, in conditions where the protein was largely interacting with the polysaccharide, 76% of its functionality was preserved after heat treatment and decomplexation (Turgeon et al., 2003). Several mechanisms have been suggested for this phenomenon, including the complex formation with a partially unfolded protein, which would restrain further aggregation (Chung, Kim, Cho, Ko, Hwang, & Kim, 2007) or the presence of a specific binding site on the protein relative to a polysaccharide (van de Weert, Andersen, & Frokjaer, 2004). Interestingly, it has been reported that the protein stabilization/destabilization effect of complexation may depend on the pH and ionic strength (Mounsey, O'Kennedy, Fenelon, & Brodkorb, 2008). It is also worth noting that the interaction between poly(lysine) and potassium pectate indicates the stereospecificity of the interaction (adoption of a superhelical conformation by pectate around the $\alpha$-helix of
poly(L-lysine) but not around poly(D-lysine) (Paradossi, Chiessi, & Malovikova, 2001).

1.8. Parameters influencing protein-polysaccharide attractive interaction

From a long list of references, it seems that the factors affecting protein-polysaccharide associative phase separation can be classified as extrinsic or intrinsic factors. Extrinsic parameters encompass all parameters not linked to the chemical/molecular composition of the biomacromolecules (Schmitt et al., 2009), including pH, ionic strength, biopolymer mixing ratio, total biopolymer concentration and processing factors (e.g. temperature, pressure, shearing rate and acidification method). Intrinsic parameters are related to the nature and the chemical/molecular properties of the interacting molecules such as the molecular weight (MW), biopolymer flexibility (ease of unfolding the native structure and/or backbone flexibility), charge density and its distribution (Tolstoguzov, 1997; Dickinson, 1998; Turgeon et al., 2009). Changes in these factors may induce biopolymer conformational changes and modifications in biopolymer-solvent interactions, two fundamental parameters in the establishment of protein-polysaccharide interactions (Doublier et al., 2000).

1.8.1. Extrinsic parameters

1.8.1.1. Influence of pH

pH has a significant role in controlling the electrostatically mediated interaction between proteins and polysaccharides due to its direct influences on the degree of ionization of the functional side groups carried by the two biopolymers (i.e. amino and carboxylic groups) (Schmitt et al., 1998, 2009; Ye, 2008). The net electrical charge on a polymer, and hence the degree to which it can become involved in
coulombic interactions, depends on how far its isoelectric point (Ip) differs from the solution pH. Most food proteins (Ip~5) can interact with anionic polysaccharides (pK$_a$~3) in the intermediate region of pH (3-5) where the two macromolecules carry opposite net charges (pK$_a$<pH<Ip) (Dickinson, 1998; Ye, 2008). It should be noted that most natural polysaccharides are anionic with the exception of chitosan and glycosaminoglycans. Consequently, the intermediate region of pH in which interaction takes place is below the Ip of the protein. For every protein-polysaccharide pair, there is a pH value for which the number of charges of opposite sign carried by the two biomacromolecules is maximum and equivalent. This pH is known as the electrical equivalence pH (EEP), where the interaction strength between the two biopolymers is the highest resulting in the maximum yield of complex formation as well as the volume of coacervate phase (Schmitt et al., 2009). Practically, the EEP is generally determined by measuring the zeta-potential ($\zeta$-potential) of the two biopolymers on the whole pH range and finding the pH value where the two $\zeta$-potential values are equal but of opposite signs (Mekhloufi et al., 2005; Guzey, & McClements, 2006). Another analytical technique is monitoring the increase of the scattered light in turbidity or light scattering experiments as a function of the pH and to determine the point of highest turbidity/scattered intensity before dropping (mainly corresponding to macroscopic phase separation) (Weinbreck, de Vries, Schrooyen, & de Kruif, 2003b; Mekhloufi et al., 2005). It is noteworthy that small variations of pH in the order of 0.5 pH unit, or even less around critical pH values, may lead to a substantial reduction of the yield of complex formation and coacervation (Schmitt, Sanchez, Thomas, & Hardy, 1999).

During a titration study of the pH influence on the structural transitions in the coacervation process (under constant conditions), two main pH values can be
identified (Schmitt et al., 2009; Turgeon et al., 2009). As a summary, the first critical pH (called pH\textsubscript{c} as determined by a slight increase in the turbidity or scattered light or hydrodynamic radius) characterizes the formation of the first electrostatic complexes that remain soluble because of uneven charge compensation. Upon further titration, a second critical pH (called pH\textsubscript{φ}) is obtained, with reference to macroscopic phase separation as determined by a sharp phase transition within the system (Schmitt et al., 2009; Turgeon et al., 2009; Schmitt, & Turgeon, 2011). Girard, Turgeon and Gauthier (2002) reported that urea addition into the mixture of β-lactoglobulin-high-methoxyl pectin decreased the pH\textsubscript{c} from 5.0 to 4.5, indicating that stronger ionic interactions were needed to induce complex formation without hydrogen bonds. In situ acidification using glucono-δ-lactone or GDL (also called the gradual pH change method) led to similar results to the classical titration method (Weinbreck et al., 2003b; Mekhloufi et al., 2005). Combination of this approach with other complementary experimental methods resulted in the identification of more critical pH values of structural transitions (Mekhloufi et al., 2005).

1.8.1.2. Influence of ionic strength

The electrostatic entropy gain induced by the release of the biopolymer counterions upon complex formation is an important driving force for the associative attraction. When the ionic strength of the system increases, two major energetically detrimental effects can occur (Fig. 1.8):

1. The screening of the charges of the macromolecules due to small ion-pairing
2. The overall equivalence of the counterion concentration in the bulk phase and in the neighbourhood of the biopolymer chains
The first effect reduces the number of protein molecules capable to interact with the polysaccharide, whereas the latter one suppresses the energetic advantage of forming a complex as already predicted by the Veis and Aranyi model. In addition, adding salt to the solvent modifies its dielectric constant which is detrimental as far as electrostatic interactions are concerned (Schmitt et al., 2009).

**Fig. 1.8:** Influence of the ionic strength on the interaction between an anionic polysaccharide and a cationic protein.

As mentioned before, counterions can shield (screen) charged groups on both proteins and polysaccharides resulting in a decrease in the strength of the interaction between the macromolecules at high salt concentration (Ye, 2008; Turgeon et al., 2009). Under such conditions, complexes dissociate completely and no phase separation occurs (de Kruif et al., 2004). At low ionic strength, the microion concentration has only a minor effect on protein–polysaccharide complexes. The number of charges present on the proteins and polysaccharides is sufficient to allow electrostatic interaction (Ye, 2008). It seems that at very low salt concentration, complexation may be suppressed (de Kruif et al., 2004) maybe due to an effective electrostatic repulsion of the non-complexed patches of the protein caused by the
non uniform charge distribution of the protein surface (Seyrek, Dubin, Tribet, & Gamble, 2003). Nevertheless, it should be noted that increasing the ionic strength to some extent by addition of small amounts of salts can in some cases have beneficial effects (Turgeon et al., 2009). Low salt content enables overcoming very short-range repulsions between the protein and the polyacid (Seyrek et al., 2003) which strengthen hydrophobic interactions as the secondarily responsible for the higher yield of complex formation (Schmitt et al., 2009; Turgeon et al., 2009). Weinbreck et al. (2004) reported that the addition of 45 mM NaCl in a whey protein-carrageenan mixture resulted in the maximum value of pHφ, indicating that the presence of monovalent ions is favorable to the formation of complexes by screening the residual negative charges of the carrageenan. Similarly, Wang, Lee, Wang and Huang (2007) reported that the addition of < 210 mM NaCl resulted in a coacervate with higher β-lactoglobulin and pectin content at the surface of a quartz crystal microbalance. The likely explanation could be related to the salting-in effect on proteins. This effect could promote exposure of new regions of the protein surface to the solvent, enabling new electrostatic interactions to occur (Schmitt et al., 2009). If divalent ions are used, suppression of coacervation occurs at lower ionic strength values than for monovalent ions, owing to double electrostatic entropy gain upon releasing two monovalent ions compared to a single divalent ion (Schmitt et al., 2009). An interesting feature is that the suppression of complex coacervation by salt addition could be overcome by dilution of the system with the solvent. The expected effect here is that the bulk ion concentration becomes lower than that of the counterions close to the biopolymers, favoring again complex formation (Schmitt et al., 2009). The general conclusion which can be obtained from the studies that considered the interplay between the ionic strength and the critical pHc and pHφ, is that for a given
protein to polysaccharide ratio, addition of monovalent ions shifted both pH values towards more acidic values in order to compensate the partial screening of the charges induced by the added microions (i.e. the charge density of proteins needs to be increased so as to reach the same level of charge neutralization between proteins and polysaccharides) (Schmitt et al., 2009). Interestingly, divalent ions addition shifted the pHφ value towards more basic pH values (up to pH 8 upon addition of 200mM CaCl₂) which can be due to the indirect complex formation between two negatively charged biopolymers via calcium bridges (Weinbreck et al., 2004). Thus, ionic strength dependence for complex formation between protein and polysaccharide might not only be related to salt concentration, but sometimes to the type of ion (Schmitt et al., 2009). Specific ions can also modulate protein denaturation and aggregation as well as induce gelation of the polysaccharide (Turgeon et al., 2003).

1.8.1.3. Influence of mixing ratio

Mixing ratio has an important effect on the characteristics and the behavior of the resulting complexes, including the complexes size, composition, and viscosity in solution because of its ability to modify the charge balance of complexes at constant pH and ionic strength (Weinbreck, Tromp, & deKruif, 2004d; Ganzevles, Cohen Stuart, van Boekel, & De Jongh, 2007; Turgeon et al., 2007, 2009; Schmitt et al., 2009). For a mixture, maximum complex formation and coacervation can be obtained at a specific ratio of protein to polysaccharide at a given set of pH and ionic strength conditions (Turgeon et al., 2009; Schmitt et al., 2009). Based on the results of Monte Carlo simulation, it has been found that these conditions exactly correspond to full charge compensation between the two macromolecules (a globular protein + a flexible polyelectrolyte) (Schmitt et al., 2009). The optimum
mixing ratio can be determined from electrophoretic mobility measurements of the two biopolymers at constant weight concentration or by measuring the turbidity and scattered light intensity of mixtures upon titration. At the complete charge neutralization ratio, the electrophoretic mobility of the complexes approaches to zero, whereas the turbidity or scattered light intensity passes through a maximum indicating a maximum mass within the electrostatic complexes and/or a maximum number of formed particles (Schmitt et al., 2009). When one of the components (protein or polysaccharide) in the mixture is in excess, complexes remained soluble because of insufficient charge neutralization (Turgeon et al., 2009; Schmitt et al., 2009). Additionally, mixing biopolymers at low protein to polysaccharide mixing ratio results in the formation of smaller complexes that remain charged over a wider pH range (Turgeon et al., 2009). At low protein to polysaccharide mixing ratio only intrapolymeric (soluble) complexes are formed, whereas at higher mixing ratio the coacervate or interpolymeric complexes volume fraction is increased (Turgeon et al., 2009). The structure of complexes or coacervates is greatly influenced by the mixing ratio: at low protein to polysaccharide mixing ratio, smaller coacervates form and coalesce rapidly into very large coacervates, whereas at higher ratio the coacervates do not coalesce as readily (Schmitt et al., 2001b). Regarding the interplay between the protein to polysaccharide ratio and the two critical pH values, pH_c and pH_φ, it is important to note that pH_c is independent from the mixing ratio. Thus, as soon as protein molecules mix with the polysaccharide ones, soluble complexes start forming, independently from the initial mixing ratio (Schmitt et al., 2009; Turgeon et al., 2009). In contrast, pH_φ, is strongly ratio dependent as it corresponds to full saturation of the polysaccharide chains by protein molecules (Kaibara, Okazaki, Bohidar, & Dubin, 2000; Schmitt et al., 2009; Turgeon et al., 2009).
1.8.1.4. Influence of total biopolymer concentration

Electrostatic complexation and coacervation can occur at a wide range of the total biopolymer concentration, starting at extremely low concentrations ($10^{-2}$ mg/ml), providing sufficiently low ionic strength conditions ($< 200$ mM), compared to the concentration needed for a segregative phase separation ($\sim 4\%$ w/w under the absence of protein aggregates) (Tolstoguzov, 1986). At too high biopolymer concentrations, when the polysaccharide or the protein is in excess in the solution, an auto-suppression of the interaction occurs, due to the entropic factors favoring complex coacervation (Weinbreck et al., 2003b; Turgeon et al., 2009). Once the mixture composition reaches the concentration of the complexed or coacervated phase, the entropy gain is completely lost and there is no driving force for the phase separation (Li et al., 1994). Indeed, increasing the total biopolymer concentration above a critical value favors the release of more counterions in solution and therefore their concentration in the dilute and in the coacervate phase. These counterions screen the charges of the biopolymers, suppressing complexation and increasing the solubility of the complexes (Weinbreck et al., 2003b, Turgeon et al., 2009). This would be the main reason why experimentally, the two-phase region is defined by a finite area in ternary phase diagrams and why complex coacervation can be induced from a monophasic system upon dilution with the solvent (Schmitt et al., 2009). Moreover, at high biopolymer concentrations, the system will show phase separation through thermodynamic incompatibility because of the competition between the macromolecules for the solvent (Tolstoguzov, 1986, 1997).

It seems that the critical concentration is highly system dependent as, for example, critical concentrations of 4.5% (w/w) and >15% (w/w) were needed to observe auto-suppression of coacervation in the $\beta$-lactoglobulin-acacia gum system
(pH 4.2) and in the whey proteins-acacia gum system (pH 3.5), respectively (Schmitt, Sanchez, Despond, Renard, Thomas, & Hardy, 2000; Weinbreck et al. 2003b). The presence of protein aggregates was noticeably broadening the auto-suppression concentration from 4.5% (w/w) to 20% (w/w) suggesting that a control of coacervation self-suppression could be obtained through tailoring the size and surface properties of aggregates (Schmitt et al. 2000). Protein aggregates can therefore be used to stabilize protein-polysaccharide coacervates from coalescence and phase separation (Schmitt et al., 2011). Regarding the interplay between the total biopolymer concentration and critical pHs, it is worth mentioning that pH_c and pH_φ are independent from total biopolymer concentration below 0.5% (w/w) (Weinbreck et al., 2003, 2003b). Nevertheless, for higher total biopolymer concentration, pH_φ has been reported to shift to higher values (Weinbreck et al., 2003b). It has been shown that the size of the coacervates is dependent on the total biopolymer concentration up to 1% (w/w) and independent of it at higher concentrations for the β-lactoglobulin-acacia gum system (pH 4.2) (Schmitt et al., 2000).

1.8.1.5. Influence of processing factors

Processing factors, including temperature, pressure, shearing and acidification rate can affect the formation and the stability of protein–polysaccharide complexes (Ye, 2008).

1.8.1.5.1. Influence of temperature

Temperature is known to possibly affect conformation of proteins and polysaccharides, but also to favor several non-electrostatic interactions (Tolstoguzov 1997; Schmitt et al., 1998). An increase in temperature enhances hydrophobic interactions and covalent bonding, whereas low temperature is favorable to
hydrogen bond formation (Dickinson, 1998; Ye, 2008; Schmitt et al., 2009). Regarding the temperature effect, there is an indicator called molar heat capacity or heat capacity change ($\Delta C_p$) which is highly sensitive to the interactions between macromolecule residues and solvent molecules (Schmitt et al., 2009; Aberkane, Jasniewski, Gaiani, Scher, & Sanchez, 2010). It can be calculated from the slope of the binding enthalpy ($\Delta H$) vs. temperature relationship using isothermal titration calorimetry (ITC) experiments (Turgeon et al., 2007; Schmitt et al., 2009). $\Delta C_p$ originates from changes in the degree of surface hydration in the free and complexed molecules, and to a lesser extent from changes in molecular vibrations (Jelesarov, & Bosshard, 1999). The different signs of the $\Delta C_p$ indicate different mechanisms of interaction between macromolecules or complexed macromolecules and solvent (Aberkane et al., 2010). A large positive value is a typical signature of ionization/charge neutralization reactions. This mainly arises because less charged groups are in contact with the solvent after complex formation. On the other hand, hydrophobic interactions produce a negative $\Delta C_p$. A positive $\Delta C_p$ but with a $\Delta H$ parameter remaining favorable (i.e. $\Delta H < 0$) at all studied temperatures, would be indicative of a significant contribution of hydrogen bonding (Gonçalves, Kitas, & Seelig, 2005; Turgeon et al., 2007). As an example, the ester groups of pectin could form hydrogen bonds with the hydroxyl, amide, phenyl and carboxylic groups of the $\beta$-lactoglobulin (Girard et al., 2002a). $\beta$-lactoglobulin-acacia gum mixed system showed a decrease in the binding enthalpy ($\Delta H$) with increasing temperature (up to 50 °C) and also a positive $\Delta C_p$, which could be attributed to hydrogen bond breakage (Aberkane et al., 2010). It has been suggested that hydrogen bonding would be involved, particularly at pHs above the proteins’ Ip (Girard et al., 2002a) and is only favored when the charge densities are low. In some mixtures (e.g. BSA and sodium
alginate at pH 6.8 and ionic strength of 0.1 M), high temperature (> 70 °C) led to conformational changes (favoring exposure of more additional hydrophobic regions) in BSA around its denaturation temperature of 55 °C, and resulted in the formation of complexes with alginate through hydrophobic interactions generated by heating which overcame electrostatic repulsions (Harding, Jumel, Kelly, Gudo, Horton, & Mitchell, 1993). No complexation occurred between 35 and 70 °C. In a mixture of two proteins (α-lactalbumin and lysozyme), Nigen et al. (2007) showed that co-precipitates can be obtained below 5 °C, whereas coacervates can be obtained at mixing temperature of 45 °C. They suggested that electrostatic interactions could be mostly important during the initial biopolymer complex formation but large-scale aggregation or coacervation would be mainly driven by hydrogen bonding or hydrophobic interactions, depending on the temperature (Nigen et al., 2007). In the β-lactoglobulin-alginate system, the possibility of the complexation was investigated in the presence of urea (to shield hydrogen interaction) or SDS (to shield hydrophobic interaction). It was found that the gelified structures formed at pH 6 were strongly influenced by hydrogen bonding and featured a balance between electrostatic attractive interactions and hydrogen bonding, the latter being principally controlled by the amount of alginate in the system (Turgeon et al., 2009). It was shown that temperature can also affect the structure of the polysaccharide (Kayitmazer et al., 2007b). In their study, the two polyelectrolytes (chitosan and polyDADMAC) had similar charge densities but the rheological properties of their coacervate phase with BSA were different, which was attributed to the stronger effect of temperature on the flexibility of chitosan. BSA-anionic polysaccharide interactions were stronger after heat denaturation because the increased molecular flexibility in the denatured state led to configurational adjustments that maximized
interactions, yielding more stable complexes than those formed with the native proteins (Samant, Singhal, Kulkarni, & Rege, 1993). Regarding the interplay between the temperature and critical pHs, it is worth noting that $\text{pH}_c$ and $\text{pH}_\phi$ are not affected by temperature as long as electrostatic interaction is the main driving force for complex formation (Kaibara et al., 2000; Weinbreck et al., 2004a).

1.8.1.5.2. Influence of pressure

High pressure (HP) treatment can be used to control the formation of protein-polysaccharide complexes due in part to a partial denaturation (e.g. increasing surface hydrophobicity) of the protein (Schmitt et al., 2009). Galazka, Smith, Ledward and Dickinson (1999) reported the formation of weak electrostatic complexes between ovalbumin and both dextran sulfate and $\iota$-carrageenan at low ionic strength conditions and pH 6.5 upon high-pressure treatment of the mixtures at 600 mPa for 20 minutes owing to exposure of more charged groups. Complexation of polysaccharide with ovalbumin at low ionic strength seems to protect the protein against pressure-induced aggregation. But, addition of 0.5 M NaCl dissociates the complexes, and the protective effect of the polysaccharide is lost (Galazka et al., 1999).

1.8.1.5.3. Influence of shearing

Shear forces can have an impact on the properties of complexes and coacervates and are an important parameter to control for industrial scale productions (Turgeon et al., 2009). Generally, shearing affects coacervation more than complexation (Schmitt et al., 2009). When a protein-polysaccharide mixture undergoes mixing (below 1000 rpm), the size of the coacervates decreases with increasing the shearing rate (Sanchez, Despond, Schmitt, & Hardy, 2001), which is mainly attributed to a breakdown of the coacervates due to interfacial destabilization.
by the shear, as is the case for emulsions, leading to fragmentation of the coacervate phase into smaller droplets. So in systems forming coacervates, finding the right conditions of temperature and shear allowed stabilization of the system against flocculation (Sanchez et al., 2001). For higher mixing rates (3500 rpm), an increase of the coacervate size was reported, probably because complex turbulent flow was favoring re-coalescence of the coacervates (Sanchez et al., 2001). Interestingly, the time of low and constant shearing rates (below 1000 rpm), has an additional effect on increasing the coacervate size maybe due to the fact that the applied shearing favors coalescence of the coacervates or is not high enough to prevent it. Combinations of two parameters including temperature and shear could either lead to a stable system (low temperature + complex shear) or to a very unstable one (high temperature + complex shear), with a marked phase separation due to the flocculation and coalescence of the coacervates (Sanchez et al., 2001). It was found that when shear forces were applied during interpolymeric complexation in a β-lactoglobulin-xanthan gum mixture, restructuring processes of insoluble complexes took place set by a competition between attractive electrostatic forces and rupture forces caused by shear (Laneuville et al., 2005).

1.8.1.5.4. Influence of acidification method

The acidification method has an effect on the formed structures. Thus, using HCl, even if diluted (0.1 M) and added slowly with enough time between drops, results in coarser complexes than those obtained with GDL, which allows for a gradual acidification. HCl application in β-lactoglobulin-xanthan gum mixed systems leads to formation of fibrous complexes, whereas when GDL is used, particulated complexes can be obtained (Turgeon et al., 2009) (Fig. 1.9).
Fig. 1.9: Phase contrast micrographs showing (a) fibrous complexes obtained by slowly adding HCl 1N to a whey protein/xanthan gum system at ratio 2:1 and 1% w/w total biopolymer concentration (pH 4), (b) Particulated complexes obtained using the same biopolymers but by gradual acidification with GDL (pH 4), reproduced from Turgeon et al. (2009).

1.8.2. Intrinsic factors

1.8.2.1. Influence of biopolymer charge density

Proteins and polysaccharides possess a large number of ionizable and other functional side-chain groups with different pK values, leading to differences in shape, size, conformation, flexibility and net charge at a given pH and ionic strength (Tolstoguzov, 1997). The charge density of the biopolymer is defined by the number of charged moieties present for a given distance along the protein or polysaccharide chain (Schmitt et al., 2009). The strength of electrostatic attractive interactions between proteins and polysaccharides and hence the type of structure to be formed depends to a great extent on the biomacromolecular charge densities as high charge density (sulphate side chains) generally leads to precipitates, whereas lower charge densities (carboxylic side chains) lead to liquid coacervates (Doublier et al., 2000; Schmitt et al., 2009; Turgeon et al., 2009; Girard et al., 2002a; Weinbreck et al. 2003b, 2003c, 2004b). This effect is attributed mainly to the fact that strong coulombic interactions induced a high compaction of the complexes with a high level
of local dehydration of the biopolymer chains, leading to insolubilization in the form of co-precipitates (Schmitt et al., 2009). It is worth mentioning that a phosphorylated polysaccharide (EPS B40) was leading to an intermediate behavior between acacia gum (carboxylated) and λ-carrageenan (sulphated) (coacervation and complexation, respectively) (Weinbreck et al., 2003). High charge density allows the formation of soluble complexes on a wider range of ionic strengths due to the local strong electrostatic interactions which are able to overcome the screening effects induced by microions (Wang, Kimura, Dubin, & Jaeger, 2000). There are great differences in the interactions of a protein with polysaccharides of different natures (e.g. sulphated such as carrageenan and carboxylated such as pectin) and also with different types of sulphated (e.g. κ, ι and λ-carrageenan) and carboxylated (low- and high-methoxyl-pectin) polysaccharides, which are related to the charge density of functional groups on the polysaccharide backbone. The interaction between oppositely charged biopolymers is enhanced when the net opposite charges of the biopolymers are increased and the ratio of net charges of the biopolymer reactants approaches unity (Ye, 2008). As an example, based on the results obtained by capillary electrophoresis, Girard et al. (2002a) reported that low-methoxyl-pectin bonded more strongly to BLG than the high-methoxyl-pectin because of having more charged carboxylic groups. The BLG-pectin system was further investigated by changing the local charge density of the pectin, so-called degree of bulkiness (Sperber et al., 2009a, 2009b). It was found that for high-methoxyl-pectins having the same degree of methylation, the one having the higher local charge density was able to form complexes with β-lactoglobulin at a higher pHc and interestingly at a higher ionic strength too. It has been shown that protein-polysaccharide complexation can occur on the wrong side of the Ip of protein (Schmitt et al., 2009; Turgeon et al.,
2009) mainly due to the presence of local high density charged regions (also called charged patches) on proteins (de Vries, Weinbreck, & de Kruif, 2003). For example, sequence analysis of the charge distribution on the surface of α-lactalbumin and β-lactoglobulin led to the conclusion that the former could bind electrostatically to acacia gum through a single patch, whereas binding of acacia gum to the latter occurred via several patches (de Vries, 2003). This could explain why α-lactalbumin was able to form electrostatic complexes more than one pH unit higher than its Ip (Weinbreck, & de Kruif, 2003). Another possible explanation of this phenomenon is that ion-dipole interaction overcomes ion-ion repulsion (Schmitt et al., 2009). Moreover, another reason would be that when the polyacid/polybase is strong enough or the protein has a high enough regulation capacity, a charge reversal may have occurred on the protein (Dickinson, 2008). The capacitance is an intrinsic property of a protein defining its ability for charge regulation on its surface (i.e. to change their charges upon interaction with a polyelectrolyte) (Schmitt et al., 2009). Monte Carlo simulation studies showed that α-lactalbumin, β-lactoglobulin and lysozyme displayed a very strong capacitance, which is stronger around their Ip (daSilva, Lund, Jonsson, & Akesson, 2006). This induces an additional and strong attraction between proteins and polyelectrolytes through charge-induced charge interactions which can in fact be stronger than ion–dipole interactions (daSilva et al., 2006).

1.8.2.2. Influence of molecular weight, molecular conformation and charge distribution

The effect of surface or linear charge density cannot be dissociated from the biopolymers structure, especially their molecular weight, flexibility and charge distribution (Doublier et al., 2000). Molecular weight and charge density play an
important role on the resulting biopolymer flexibility (Kayitmazer, Shaw, & Dubin, 2005). The increase of the molecular weight of the polysaccharide favors the formation of electrostatic complexes. Indeed, the occupied volume of the polysaccharide increases with increasing molecular weight, enabling a higher number of proteins to interact with it and to build complexes (Schmitt et al., 2009). Regarding the interplay between the polyelectrolyte molecular weight and the charge density, Shieh and Glatz (1994) reported that an increase in the molecular weight of the polyelectrolyte (polyacrylic acid) led to much stronger binding of ovalbumin due to a large difference between their charge densities. However, this effect was not observed with lysozyme which has the same charge density as polyacrylic acid. Studies on polyDADMAC (a strong cationic polymer) of various molecular weights, interacting with anionic surfactant micelles, showed that an increase in the molecular weight of the micelle reduced the required charge density for coacervation and increased the coacervation yield (Wang et al., 2000). Furthermore, they concluded that a critical molecular weight of the polycation existed for every total polymer concentration and mixing ratio tested. This critical molecular weight was leading to a critical size of 45 nm for the complexes. Below this boundary, coacervation was not possible (Wang et al., 2000). Therefore, by adjusting the size of the micelles or the polyelectrolyte, the final size of the complexes/coacervates can be controlled (Turgeon et al., 2009). Laneuville, Sanchez, Turgeon, Hardy and Paquin (2005b) reported the influence of the molecular weight of xanthan gum on the size and compactness (fractal dimension, $d_f$) of the formed electrostatic complexes. Lower molecular weight resulted in smaller and denser ($d_f \sim 2.56$) complexes, whereas high molecular weight xanthan gum led to much larger and more linear ($d_f \sim 2.26$) complexes. Moreover, the internal structure of the complexes can be modulated by
varying the molecular weight of the polysaccharide, thus allowing additional control of their properties (Laneuville et al., 2005b). It is worth noting that, at a given ionic strength, small molecular weight polyelectrolytes generally led to smaller soluble (primary) complexes, whereas larger ones induced further aggregation of the soluble complexes leading ultimately to interpolymeric complexes (Li et al., 1994; Wang et al., 2000; Laneuville et al., 2005b), presumably since the polyelectrolyte acts as the backbone for the formation of primary complexes and possibly also due to a higher entropy gain (Laneuville et al., 2005b, Turgeon et al., 2009). The presence of large protein aggregates also influences coacervation (Schmitt et al., 2001b): protein aggregates are not able to form coacervates, instead they precipitate and form structures composed of a protein core surrounded by a polysaccharide layer. Thus, samples containing aggregates will present different functional properties.

The number of proteins bound to a polyelectrolyte depends largely on the polymer contour length, chain flexibility, and protein dimensions. It has been suggested that a more compact conformation of the polyelectrolyte results in a higher charge density and therefore larger polarizing effect on the protein, which promotes a stronger charge-induced interactions (Turgeon et al., 2009). It has also been reported that flexible proteins (e.g. caseins or gelatin) bind polysaccharides more strongly than globular proteins (e.g. BSA or BLG) and that the thermal denaturation of the latter enhances their binding affinity. The likely explanation is that flexible molecules are able to form a maximum number of contacts (junction zones) with the other oppositely charged molecules (i.e. an increase in local concentration of interacting groups is favored) (Doublier et al., 2000). It seems that low charge density polysaccharides only interact with proteins if they adopt a more charged ordered conformation and not a less charged disordered conformation (helix and
random coil conformations, respectively) (Doublier et al., 2000). However, in order to obtain coacervation, molecules should be in a random coil configuration (Burgess, 1990). It seems that the increased chain flexibility and charge mobility resulting in stronger binding are more important for micelles than for single proteins (Kayitmazer, Seyrek, Dubin, & Staggemeier, 2003). Because of the inverse relationship between biopolymer charge density and flexibility, one may hypothesize that the optimum interaction between proteins and polysaccharides would occur at a critical balance between these two parameters (Doublier et al., 2000). Since the length of the binding segment is controlled by the intrinsic stiffness of the polymer chain, this parameter has a greater influence than the polyelectrolyte linear charge density (Mattison, Dubin, & Brittain, 1998).

The charge distribution can also play an important role by affecting the interaction affinities (Kayitmazer et al., 2003). As an example, ribonuclease and lysozyme are proteins with similar molecular weight and the same number of basic groups. However, their interactions with polyelectrolytes are different due to the different distribution of their basic residues. Ribonuclease has its basic residues located relatively close to one another, whereas lysozyme has its residues randomly distributed. This affected the polarizability of the resulting primary soluble complex, i.e. the soluble complexes formed with lysozyme were more prone to aggregation due to a superior polarizability mainly attributable to the lack of complementarities in the charges spacing (Takahashi, Kubota, Kokai, Izumi, Hirata, & Kokufuta, 2000). Field-theoretic simulation results have shown remarkable differences between the phases formed with uniformly or unevenly charged chains bearing the same charge density. Uniformly charged chains formed large homogeneous phases (a dilute phase and a coacervate phase), whereas the unevenly charged polyelectrolyte
(block copolymer) formed a mesophase with micellar structure (Popov, Lee, & Fredrickson, 2007).

The advances in understanding the effects of the extrinsic and intrinsic factors on protein-polysaccharide complexation endows the food scientist with the necessary tools to be able to choose the adequate biomacromolecules and experimental conditions to control the reaction kinetics and the structuration process to form the desired structures and obtain mixed systems with tailored functionalities for specific applications such as a texture agent, an emulsifier or foaming stabilizing agent, and even for the encapsulation of ingredients (Schmitt et al., 2009; Turgeon et al., 2009).

1.9. Thermodynamic background, theoretical developments and energetics of the formation of protein-polysaccharide complexes and coacervates

Generally, the main difference between thermodynamic incompatibility and complex coacervation is that thermodynamic incompatibility is predominantly entropically driven, whereas complex coacervation is both entropically and enthalpically driven (Turgeon et al., 2003). Given this general consideration, let us review data obtained for protein–polysaccharide associative systems. The formation of protein-polysaccharide complexes takes place spontaneously when the total electrostatic free energy of the mixed biopolymer solution ($G$) decreases (i.e. $\Delta G<0$) regardless of the actual amount of favorable free energy change accumulated by direct molecular contact between the associating macromolecules (Tolstoguzov, 1997; Jelesarov et al., 1999; de Vries et al., 2006; Dickinson, 2008). A delicate balance between entropic ($-T\Delta S$) and enthalpic ($\Delta H$) contributions determines the value of $\Delta G$, and therefore the possibility or not for biopolymers to form a complex
(Turgeon et al., 2007). The entropic contribution arises from the release of counterions and occluded water molecules from both the protein and polysaccharide due to the compaction of biopolymers resulting in increasing the entropy of mixing biopolymers and their co-solubility. The enthalpic contribution mainly results from the electrostatic interactions between the biopolymers (Jelesarov et al., 1999; Schmitt et al., 2009) as well as from the solvent reorganization in the solution (Li et al., 1994; Tolstoguzov, 1997; de Kruif et al., 2004; Turgeon et al., 2007). It should be noted that the compaction of the protein and the polysaccharide within a complex also results in an unfavorable loss of configurational entropy (i.e. reduced mobility) of the macromolecules. In addition, the ordering of water molecules at the complex interface may contribute unfavorably to the entropy of the system (Jelesarov et al., 1999; Dickinson, 2008). Due to the complexity of the water structure and the subtlety of the processes involved, it is difficult to estimate the total entropic contribution (Tolstoguzov, 2001).

In order to get a better understanding of the energetics of complex formation and coacervation in protein-polysaccharide systems and to challenge theoretical models with experimental data, a number of calorimetry-based studies (using differential scanning and isothermal titration calorimetry methods) have been conducted during the last decade (de Kruif et al., 2004; Turgeon et al., 2007). In fact, the situation appears more intricate than expected resulting in contradictory claims. Generally, it seems that the obtained calorimetry results depend markedly on the systems considered, as both exothermic and endothermic signals have been recorded. For example, exothermic signals (indicating the enthalpic contribution) have been recorded upon titration of BLG with acacia gum, low- and high-methoxyl-pectins and sodium alginate (Girard, Turgeon, & Gauthier, 2003b; Schmitt et al.,
2005; Harnsilawat, Pongsawatmanit, & McClements, 2006) or upon titration of α-lactalbumin with lysozyme (Nigen et al., 2007). In contrast, calorimetry data reporting complex formation between proteins and strong polyelectrolytes showed endothermic signals which indicate the entropic contribution (Feng, Pelton, Leduc, & Champ, 2007). In addition, it was also shown that the heat of binding recorded by ITC displayed a complex pattern with the successive appearance of an exothermic and endothermic signal (Ziegler, & Seelig, 2004; Gonçalves et al., 2005; Harnsilawat et al. 2006; Aberkane et al., 2010), and even of the appearance of a simultaneous exothermic – endothermic signal (Nigen et al., 2007). The occurrence of an endothermic signal following the exothermic one was shown to be induced by condensation of complexes (Ziegler et al., 2004; Gonçalves et al., 2005) or coacervation (Harnsilawat et al., 2006), indicating the secondary involvements of additional short-range interactions such as hydrophobic, van der Waals forces or hydrogen bonds (Schmitt et al., 1998). It should be noted that water molecule release and changes in biopolymer conformation could also play a role (Turgeon et al., 2007). These different behaviors can be explained taking into account the “condensation” phenomenon displayed by oppositely charged polyelectrolytes (Turgeon et al., 2007). If the distance (a) between two charges is shorter than the Bjerrum length above which thermal agitation is higher than electrostatic attraction, (l_B), a large fraction (1-a/l_B) of counterions is trapped close to the polyion. During complex formation, the charge neutralization of oppositely charged species can lead to a release of condensed counterions that regain as much translational entropy as the free ones. Now, such effects can be supposed to be very different between a strongly charged polyelectrolyte, where condensation is important, and weakly charged polyelectrolytes such as proteins and most polysaccharides where
condensation is negligible (Gummel, Cousin, & Boue, 2007). Based on the Langevin dynamics simulation, Ou and Muthukumar (2006) described two scenarios for the energetics of polyelectrolyte complexation in different salt concentrations depending on whether the polyelectrolyte is weak or strong. It was shown that for weaker polyelectrolytes, a more favorable reduction of the enthalpy (negative enthalpy change) due to electrostatic attraction represents the driving force for complex formation leading to an exothermic signal, while the entropy gain due to counterion release plays only a minor role (Girard et al., 2003b; Ou et al., 2006). So, weakly charged biopolymers tend to associate through direct enthalpic electrostatic interactions, whereas the formation of aggregated complexes is entropically driven, as a consequence of the conformational changes of the biopolymers and the release of counterions and water molecules into bulk solution (Turgeon et al., 2007). On the other hand, complex formation between highly charged polyelectrolytes is mainly entropically driven owing to the release of the condensed counterions via the ion-exchange process and opposed by a positive enthalpy change (Schmitt et al., 2011) leading to an overall endothermic signal (Ou et al., 2006; Schmitt et al., 2009; Turgeon et al., 2009). This assumption is supported by the strong ionic strength dependence of highly charged polyelectrolyte complexation (suppression of complex formation above a critical ionic strength) resulting in the disappearance of the entropic gain (counterion concentration being more or less equivalent in the bulk phase and in the neighborhood of the biopolymers) (Seyrek et al. 2003; Weinbreck et al., 2003b). Counterion release has been studied by computer simulations (Ou et al., 2006). Gummel et al. (2007) were the first to demonstrate experimentally the release of counterions following lysozyme-polystyrene sulfonate complexation using
neutron scattering and a specific labeling technique utilizing deuterated tetramethyl-
ammonium counterion.

Several theories describing protein-polysaccharide complexes and coacervates were developed in the late 1950s. They were based mainly on data obtained for the gelatin-acacia gum or gelatin A-gelatin B mixed systems (Overbeek and Voorn 1957; Veis and Aranyi 1960). Besides the assumption that the polyelectrolytes had a random coil configuration, that the solvent-solute interactions were negligible and that the interactions were not site-specific, Overbeek and Voorn (1957) clearly explained the main balance of forces: complex coacervation as a spontaneous mechanism is mainly driven by a decrease in the electrostatic free energy (which has both enthalpic and entropic contributions), and is opposed by macro-ion configurational and translational entropy. Using the same approximations, Nakajima and Sato (1972) slightly modified this theory taking into consideration the effect of added salt in the coacervate phase. In order to correct for the rather poor agreement of the Voorn-Overbeek theory with obtained data on mixed (salt free) gelatin systems, Veis and Aranyi (1960) included the effects of non-electrostatic interactions into the Voorn–Overbeek theory. The theory of Veis and Aranyi takes also into account solvent-solute interactions and considers complex coacervation as a two-step process rather than a spontaneous one. Hence, the first step leads to the formation of aggregates resulting from charge neutralization between the polyelectrolytes of low configurational entropy. In a second step, these aggregates (or complexes) rearrange into a coacervate phase in order to increase their configurational entropy. This model assumes also the presence of ion-paired aggregates in the equilibrium dilute phase. An important characteristic of this model is that polyelectrolyte-solvent interactions are weak at low total polyelectrolyte
concentration but stronger at high total polyelectrolyte concentration, accounting in part for the observed self-suppression of complex coacervation. Later on, Tainaka (1979) slightly adapted the Veis-Aranyi model by introducing restrictions on the charge density of the polyelectrolytes and on their molecular weight. Experimental evidence tends to support that the Veis and Aranyi model is probably the best at describing the entire phase separation phenomenon (Schmitt et al., 2009). Recently, Allen and Warren (2004) developed an elegant self-consistent field (SCF) theory for complex coacervation of weakly charged flexible chains and weakly charged spherical macro-ions. Given the known limitations of SCF theories of polyelectrolyte adsorption, the quantitative validity of this theory is presumably restricted to coacervate phases that are rather dilute (much more dilute than 10% (w/w)).

1.10. Kinetics of phase separation

The fundamental question concerning the dynamic mechanism of complex coacervation is how weak non-specific interactions between proteins and polysaccharides lead to self-organization of the system resulting in the formation of liquid droplets ranging from hundreds of nanometers to several microns in diameter (Kaibara et al., 2000; Schmitt et al., 2000, 2001b; Turgeon et al., 2003). As mentioned previously, these coacervates tend to coalesce to form ultimately a dense liquid phase, (Schmitt et al., 2009). By nature, phase separation in protein–polysaccharide systems is a dynamic process. It arises from local fluctuations of the biopolymer concentration within the entire volume of the mixture. These fluctuations can result from inefficient mixing of the components, temperature fluctuations or (non)-specific interactions (Turgeon et al., 2003). The general models conveniently used to describe phase separation kinetics are nucleation and growth (NG) or
spinodal decomposition (SD). The former is characterized by initial short-range/high-amplitude biopolymer concentration fluctuations, induced by electrostatic complex formation, which lead to hydrodynamically driven coalescence and formation of sharp interfaces delimiting the coacervate phase from the dilute phase (Turgeon et al., 2003; Sanchez, Mekhloufi, & Renard, 2006); whereas SD proceeds through long-range/small-amplitude fluctuations (Turgeon et al., 2003). NG generally ends up with spherical droplets dispersed in a continuous phase, whereas SD exhibited a 3D interconnected network (Turgeon et al., 2003). The coalescence of the coacervates can be explained by the reduction of the interfacial energy of the system because of the appearance of interfacial viscoelasticity in the phase concentrated in biopolymers (Schmitt et al., 2009). The coacervate phase is mainly liquid in nature as it contains around 70% water (Schmitt et al., 2000; Weinbreck et al., 2004d). The coalescence phenomenon was shown to occur very quickly (few minutes), especially in optimum conditions of pH and protein to polysaccharide ratio (Schmitt et al., 2001b). Over time, the coacervates tend to repel the water in order to increase their free energy as demonstrated by the transition from a turbid system (because of light scattering from the water inclusions) to a clear viscous and dense phase (Weinbreck et al., 2004b). Since phase separation produces a heterogeneous mixture characterized by at least two different refractive indexes, small angle light (or neutron) scattering, turbidity measurements, diffusing wave spectroscopy (DWS) and microscopic techniques (such as CLSM) are methods of choice for time-resolved analysis in order to follow the evolution of the interaction and the internal structure of the developing complexes (Turgeon et al., 2009). These methods have been actually used to determine whether a protein-polysaccharide associative phase separation could be described by a SD or NG mechanism, or both in sequence. To possibly answer this
question, small angle light scattering measurements as a function of acidification time were carried out on β-lactoglobulin–xanthan (Laneuville et al., 2005b), β-lactoglobulin–pectin (Girard et al., 2004) and β-lactoglobulin–acacia gum (Sanchez et al., 2006) systems. In β-lactoglobulin–xanthan mixture, loose and highly tenuous aggregates (primary or soluble complexes) with $d_f \sim 1.80$ formed at pH$_c$, indicating a diffusion-limited cluster aggregation mechanism (DLCA) (Laneuville et al., 2005b). This type of mechanism occurs when repulsive forces are negligible, and the colloidal aggregation model assumes that every collision results in particles sticking irreversibly upon contact (Turgeon et al., 2009). Therefore, this was interpreted as a clear signature of a NG mechanism. In the β-lactoglobulin–pectin system a clear induction time was observed, in which the size of the emerging structures decreased, indicating the formation of a large quantity of small primary complexes while the turbidity increased drastically, demonstrating that complexes also phase separated following a NG mechanism (Girard et al., 2004). A correlation peak at a fixed scattering wave vector, $q_{\text{max}}$, was recorded in β-lactoglobulin-acacia gum system after phase separation occurred (Sanchez et al., 2006). Although a correlation peak was often ascribed to a SD mechanism, the temporal evolution of $q_{\text{max}}$ rather followed that usually observed during NG. So, NG mechanism was proposed as a general mechanism of complex formation/coacervation between biological macromolecules (Sanchez et al., 2006).

1.11. Functional properties of protein-polysaccharide complexes and coacervates

The formation of non-covalent electrostatic complexes between proteins and polysaccharides can potentially lead to different functional properties, compared to
the two biopolymers taken individually (Schmitt et al., 1998; McClements, 2006). This is generally due to a synergistic combination of the functional features of both the protein (generally hydrophobic and/or hydrophilic and globular) and the polysaccharide (generally hydrophilic and branched) (Schmitt et al., 2009). Complex coacervation is a green technique for food applications as this technique neither uses organic solvents nor requires high temperatures (Schmitt et al., 1998). In recent years, many researchers have focused on the functional applications of protein-polysaccharide complexation/coacervation including encapsulation and delivery ability (Champagne, & Fustier, 2007; Gunasekaran, Ko, & Xiao, 2007; Benichou, Aserin, & Garti, 2007), viscosifying and gelling ability (Weinbreck et al., 2004d; Weinbreck, Wientjes, Nieuwenhuijse, Robijn, & de Kruijf, 2004e), foaming ability (Schmitt et al., 2005), emulsion stabilization (Neirynck, Van lent, Dewettinck, & van der Meer, 2007), protein stability and solubility in acidic dairy products (Matia-Merino, Lau, & Dickinson, 2004), separation and purification of proteins (Montilla, Casal, Moreno, Belloque, Olano, & Corzo, 2007), lightening agents (Jones, & McClements, 2010), design and development of new biomaterials (e.g. texturizing agents such as fat replacers (Laneuville et al., 2005a; Liu, Xu, & Guo, 2007) by mimicking some of the desirable characteristics of the lipid droplets, such as appearance, mouth feel, texture and optical properties, edible films (Kester, & Fennema, 1986; Shih, 1994; Park, Daeschel, & Zhao, 2004) for food protection and packaging in the food industry as well as to confer a very high potential for antimicrobial activity) and involvement in some in-mouth fat perception attributes (e.g. the flocculation of a lysozyme-stabilized emulsion was due mainly to the interaction with the glycosylated saliva mucin proteins (Silletti, Vingerhoeds, Norde, & Van Acken, 2007)). In this section, due to the general objectives of the current
study, the emphasis will be on the encapsulation and delivery properties of protein-polysaccharide associated phases.

The remarkable interfacial activity of protein–polysaccharide complexes and coacervates represents a method of choice for designing micro- or nanoparticles or different types of emulsions (simple, multiple, layer-by-layer, etc.) during a liquid/liquid phase separation (Schmitt et al., 2009, 2011). These systems are suitable for the protection of bioactive nutrients against processing (heat, redox potential, shear, etc.) and storage (temperature, light, oxygen and moisture) conditions (Ducel, Richard, Saulnier, Popineau, & Boury, 2004; Weinbreck, Minor, & de Kruiif, 2004a), for masking compounds with unpleasant aftertaste or odor (Junyaprasert, Mitrevej, Sinchaipanid, Boonme, & Wurster, 2001; Lamprecht, Schafer, & Lehr, 2001; Xing, Cheng, Yang, & Ma, 2004, 2005; Pierucci, Andrade, Farina, Pedrosa, & Rocha-Leao, 2007) and for the controlled release of various encapsulated materials of liquid and/or solid nature, enabling delivery of the material with the optimal kinetics (in the food or the mouth for the flavor) and to specific gastrointestinal targets (for bioactives and probiotics) (Yeo, Bellas, Firestone, Langer, & Kohane, 2005; Prata, Menut, Leydet, Trigo, & Grosso, 2008; Schmitt et al., 2011). Encapsulation conditions should be gentle for sensitive compounds and the release can be controlled by different kinds of triggers such as mechanical stress (chewing), temperature, pH changes (acidic conditions in the stomach, neutral in the intestine), time, osmotic force or enzymatic activity (Schmitt et al., 2011). Generally, for encapsulation, the protein and polysaccharide solutions are mixed at a pH above the Ip of the protein, with the pH of the mixture being readjusted to a value below the Ip of the protein after mixing (Ye, 2008). The coacervated phase can spontaneously form a coating layer around each dispersed particle (e.g. emulsion droplets) in the
solution media (Gouin, 2004; Weinbreck et al., 2004a). Agitation allows limiting coacervate sedimentation and assures a homogeneous coating. It is worth mentioning that two methods of encapsulation can be followed for food applications including interfacial complex coacervation or layer-by-layer deposition around an oil phase. Formation of a solid film around emulsion droplets containing the product to be encapsulated leads to the formation of microcapsules having diameters ranging from 1 to 50 µm (Lamprecht et al. 2001; Weinbreck et al., 2004a), whereas the entrapment of the solvent molecules in the coacervated phase leads to the formation of stable sub-micrometer hydrogels (Weinbreck et al., 2004a; Chen, & Subirade, 2005; Hong, & McClements, 2007). The gelatin-acacia gum system was the first to be reported for encapsulation purposes and certainly the most studied due to the unique gelling-melting profile of gelatin allowing production of vehicles able to release aroma upon cooking of the product. Another key characteristic beneficial for numerous applications is the formation of a viscous coacervate during microcapsule formation at 50–60 ºC (a temperature higher than the gel point of gelatin) resulting in better stability, a stable rigid and gelled shell around the microcapsules after cooling and easy disruption of microcapsules because of melting of the gelatin in the mouth for delivery purposes (Schmitt et al., 2009, 2011). Yeo et al. (2005) used gelatin-gum Arabic coacervates to encapsulate and monitor the release of a flavor oil under the effect of temperature as a trigger (i.e. during heating) in frozen baked goods. The oil remained stable for 4 weeks of storage at 4 and -20 ºC but was released at a temperature around 100 ºC or after exposure to 100 mM NaCl at room temperature. Interestingly, the released oil could be re-encapsulated upon cooling. The addition of surfactants was proposed to improve encapsulation efficiency (Mayya, Bhattacharyya, & Argillier, 2003), decrease droplet size and accelerate coacervation
in gelatin-gum Arabic systems (Tan et al., 2008). Weinbreck et al. (2004a) reported that a matrix of whey protein and gum Arabic can be used for encapsulation purposes. In their study, capsules containing lemon and orange flavors were prepared successfully using complex coacervation of whey protein and gum Arabic under specific conditions. Parameters such as pH, biopolymer concentration and capsule size were investigated. Capsules prepared at pH 4.0 (the pH at which the viscosity of the complex was maximum and most of the biopolymers were in a complexed form (Weinbreck et al., 2004e)) showed a smooth shell of whey protein and gum Arabic coacervate around the oil droplets. An interesting fact was that a very high payload (amount of oil in the capsule), up to 90%, could be obtained. Limonene-loaded capsules of different droplet size were introduced into a model Gouda cheese. After 1 month, the flavor release was higher for large capsules (> 50 µm), probably due to more easy disruption during chewing compared to small capsules, exhibiting the ability of modulating the flavor release. Regarding the application of soluble electrostatic complexes in the encapsulation field, Benichou et al. (2007) reported that soluble complexes based on whey protein isolate-xanthan gum could be used to stabilize multiple emulsions (W/O/W) for the controlled release of thiamine. Bedie, Turgeon and Makhlof (2008) have used whey protein isolate-low methoxyl pectin interpolymeric (insoluble) complexes to evaluate their functionality as an entrapment matrix for thiamine in acidic foods. In their study, two methods for complex preparation were used including pre-blending acidification (mixing method) and post-blending acidification (titration method). Entrapment (loading) efficiency (percent of thiamine entrapped) was higher for complexes obtained by the mixing method which was due to the formation of larger complexes during the rapid mixing of both solutions pre-adjusted to the target pH. The post-blending acidification
method led to smaller and more homogeneous complexes that should be preferred for applications in liquid foods in which sedimentation is a concern (Bedie et al., 2008). Protein-polysaccharide complexes and coacervates can be utilized as a promising alternative technique to protect probiotics from external factors such as oxidative stress during storage, bacteriophage and harsh acid stress in the upper digestive tract to then be released in the large intestine, in which their functional properties are necessary (Heidebach, Först, & Kulozik, 2010). Oliveira, Moretti, Boschini, Baliero, Freitas, and Favaro-Trindade (2007) investigated the stability of microcapsules containing *Bifidobacterium lactis* and *Lactobacillus acidophilus*, produced by complex coacervation between casein and pectin, against spray drying process, a shelf-life of 120 days at 7-37 °C and the in vitro tolerance after being exposed to low pH (1.0 and 3.0) solutions. The process used and the wall material were efficient in protecting the micro-organisms. However, microencapsulated *Bifidobacterium lactis* lost its viability before the end of the storage time.
2. Objectives of the current study

Recently, some classes of the chemical compounds such as minerals (Fe$^{2+}$, Mg$^{2+}$), antioxidants (tocopherols, flavonoids, phenolic compounds), carotenoids (β-carotene, lycopene, lutein, zeaxanthin), vitamins (D, thiamin, riboflavin), fatty acids (omega 3, conjugated linoleic acid), phytosterols (stigmasterol, β-sitosterol, campesterol), fibers (inulin), prebiotics and probiotics have been the focus of research. After isolation from a food matrix such compounds are called ‘nutraceuticals: the link between nutrition and medicine’, a term coined by DeFelice in 1979 and defined as ‘food or parts of food that provide medical or health benefits, including the prevention and treatment of disease.’ The value of these traditional supplements led to their application for food enrichment and fortification and ultimately to develop new functional foods (such as omega 3-enriched dairy products, ferrous-enriched cereal products) to prevent coronary heart disease, immune response disorders, stroke, cancer, urinary tract disease, macular degeneration and cataracts as well as to improve weight gain, mental health, bone health and visual acuity (McClements et al., 2009; Livney, 2010).

Most of the nutraceuticals show instability against chemical or physical degradation and tend to degrade during storage when incorporated into foods. Encapsulation systems, also known as ‘delivery systems’, are typically used to incorporate them into the foods (Shimoni, 2009). So, the development of structured delivery systems for the encapsulation of bioactives is an important area of research for the food industry in order to improve the quality of foods and beverages (Matalanis, Jones, & McClements, 2011). Due to the hydrophobic nature of the most functional compounds, their incorporation into aqueous foods and beverages (especially transparent ones) is challenging (Sagalowicz, & Leser, 2010; Matalanis et
al., 2011). Since there are only a limited number of food grade ingredients which can be used as the encapsulation materials, finding suitable delivery systems is of importance (Sagalowicz et al., 2010). Different delivery systems have been used to introduce bioactives into foods including oil in water (O/W) ordinary and multilayered emulsions (Shaw, McClements, & Decker, 2007; Yoksan, Jirawutthiwongchai, & Arpo, 2010), double emulsions (O’Regan, & Mulvihill, 2010), microemulsions (Flanagan, & Singh, 2006), nanoemulsions (Qian, Decker, Xiao, & McClements, 2012), solid lipid nanoparticles (SLNs) (Trombino, Cassano, Muzzalupo, Pingitore, Cione, & Picci, 2009), cyclodextrins (Choi, Ruktanonchai, Min, Chun, & Soottitantawat, 2010), amylose (Zabar, Lesmes, Katz, Shimoni, & Bianco-Peled, 2010), liposomes (Takahashi, Inafuku, Miyagi, Oku, Wada, Imura, & Kitamoto, 2007), and micelles (Semo, Kesselman, Danino, & Livney, 2007). Although some systems could protect the nutraceuticals against chemical degradation as well as showed controlled release properties, the main limitations were poor loading capacities, inability to control the polymorphism of the lipid based carriers, poor physical stability, high cost, off-taste, formation of opaque (non-transparent) systems and using materials not considered as GRAS (generally recognized as safe) (Sagalowicz et al., 2010).

The use of biopolymers such as proteins and polysaccharides to produce delivery systems in order to protect these bioactives and to enable subsequent fortification of liquid and transparent food systems (for example some fruit (such as pomegranate or apple) juices, traditional herbal beverages containing water, sugar, citric acid and herbal (such as spearmint) extracts and non-fermented and flavored malt beverages) is a highly desirable and promising technique. As mentioned previously, among the formed structures, coacervates can be used as delivery
systems for encapsulation purposes. Interaction between coacervates leads to coalescence and formation of transient multivesicular structures that can coalesce further and eventually completely separate into a dense coacervated phase (Sanchez et al., 2002), which limits its application as a delivery system in fortification of liquid foods where a uniform structure is a concern. During recent years, some researchers have examined the potential applications of soluble and/or insoluble complexes arising from protein-polysaccharide interactions in order to encapsulate hydrophilic and lipophilic molecules (Bedie et al., 2008; Zimet, & Livney, 2009; Ron et al., 2010).

The hypothesis of the current study is that the binding properties of β-lactoglobulin (a member of the lipocalin protein family) towards some hydrophobic and hydrophilic compounds can be used to produce green delivery systems in order to fortify liquid and transparent acidic food products. The produced delivery system can be protected by deposition of an anionic polysaccharide as a secondary layer (or shell) around the protein core in the pH range between the pK_a of the polysaccharide and the Ip of β-lactoglobulin to form a core-shell structure.

The successful development of nutraceutical containing delivery systems for liquid products depends on several factors: (i) the ability to disperse bioactive ingredients into an aqueous phase, in case the actives are water insoluble, (ii) the stability of the ‘capsule’ structure, preventing effects like creaming or sedimentation, (iii) minimizing the impact on the textural, rheological or optical properties of the final food product, (iv) protection of the encapsulated active molecules against degradation during processing and storage, and (v) controlled release during consumption, either in the mouth or during digestion in the gastrointestinal tract (Sagalowicz et al., 2010).
Most challenging in practical applications seems to be the sufficient stabilization of the bioactive molecules. Therefore, the **main objective** of this research project is to assess the stabilization efficiencies of β-carotene, vitamin D₂, curcumin and folic acid (as nutraceutical models) in an acidic (pH 4.25) transparent beverage model using nanoparticles (soluble complexes) produced via electrostatic interactions between β-lactoglobulin and anionic polysaccharides (including sodium alginate, gum Arabic, κ-carrageenan and carboxymethyl cellulose). The **specific objectives** of this research project are:

- To assess the nature of the interactions between β-lactoglobulin and the above-mentioned anionic polysaccharides
- To assess the effect of polysaccharide sonication on the properties of the produced complexes
- To assess the effect of pH on the binding ability of β-lactoglobulin to nutraceutical models
- To assess the binding and diffusion of the nutraceutical models using NMR

As mentioned previously the task to find the appropriate delivery system is more challenging for the food industry compared to other fields such as pharmacy, medical products or cosmetics, since only a limited amount of ingredients (of food grade quality) can be used as encapsulation and stabilization material (Sagalowicz et al., 2010). Fortification of liquid products, such as drinks and juices is getting more and more fashionable in foods. In this case, the encapsulated active ingredients must be stabilized in a liquid environment, which is considerably different from stabilizing the active ingredients in a solid environment (Sagalowicz et al., 2010). Solid microcapsules or powders prepared from spray drying processes cannot be
simply added to an aqueous food product due to losing the barrier and stabilization function of the solid capsule shell material. When adding the solid microcapsules into water, the capsule shell or matrix material is basically dissolved into the aqueous phase releasing the active ingredients into the bulk phase and, in general, protection is lost. Therefore, delivering active ingredients in a liquid matrix is by far more challenging than the delivery of the active ingredients in a solid phase and requires the use of different encapsulation and protection strategies. Table 2.1 summarizes the various types of systems available for the delivery of bioactive ingredients in liquid food products (Sagalowicz et al., 2010).

It seems that the main activity in the research field of aqueous delivery systems is to try to physically or chemically ‘complex’ or ‘bind’ the active ingredients (specially hydrophobic ones) to a molecular or supramolecular structure with the hope to protect it in this way from chemical or physical deterioration (Sagalowicz et al., 2010). Indeed, many types of biopolymers are capable of binding lipophilic and hydrophilic molecules and forming molecular complexes. The active molecules may be bound to individual biopolymer molecules, or they may be incorporated within clusters formed by a single type or mixed types of biopolymers (Matalanis et al., 2011).
Table 2.1: Various types of systems available for the delivery of bioactive ingredients in liquid food products

<table>
<thead>
<tr>
<th>Delivery system</th>
<th>Size</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
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<tbody>
<tr>
<td>Powder particles</td>
<td>10 μm – 1 mm</td>
<td>- Good encapsulation for solid food products</td>
<td>- Hardly adapted for delivery in liquids</td>
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<td>- Glass encapsulation</td>
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<tr>
<td>- Core-shell capsules</td>
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<td>- Matrix capsules</td>
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<tr>
<td>Oil in water emulsions</td>
<td>100 nm – 10 μm</td>
<td>- Host lipophilic molecules</td>
<td>- Physical stability sometimes an issue</td>
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<tr>
<td>- Ordinary emulsions</td>
<td></td>
<td>- Better chemical protection of sensitive oil achieved when multilayered emulsion or SLNS used</td>
<td>- Polymorphism stability and encapsulation for SLNS difficult to control</td>
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<tr>
<td>- Multilayered emulsions</td>
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<td>- Double emulsions</td>
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<td>- Nanoemulsions</td>
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<td>- Solid lipid nanoparticles</td>
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<tr>
<td>Molecular complexes</td>
<td>10 nm – 600 nm</td>
<td>- Solubilization of small lipophilic molecules</td>
<td>- Loading capacity may be limited</td>
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<tr>
<td>- Cyclodextrin</td>
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<td>- Protection of sensitive molecules</td>
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<tr>
<td>- Amylose</td>
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<td>- Removal of cholesterol</td>
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<td>- Proteins</td>
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<tr>
<td>- Protein aggregates</td>
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<tr>
<td>Liposome (Vesicles)</td>
<td>20 nm – 100 μm</td>
<td>- Solubilization of hydrophilic and lipophilic molecules</td>
<td>- High cost (ingredients and process)</td>
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<tr>
<td></td>
<td></td>
<td>- Sustained release of nutrients</td>
<td>- Poor loading efficiency and capacity</td>
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<td>Oil in water microemulsions</td>
<td>5 nm – 100 nm</td>
<td>- Solubilization of lipophilic molecules</td>
<td>- Large amount of surfactant needed</td>
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<td></td>
<td></td>
<td>- Solubilization of crystallizing molecules</td>
<td>- Often off-taste</td>
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<tr>
<td></td>
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<td>- Increase in bioavailability</td>
<td>- Used surfactants often not well accepted</td>
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<td>- Transparent appearance</td>
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<tr>
<td>Dispersed reversed surfactant systems</td>
<td>100 nm - 1 μm</td>
<td>- Solubilization of amphiphilic and lipophilic molecules</td>
<td>- Large amount of surfactant may be needed</td>
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<tr>
<td>- Cubosomes, Hexosomes</td>
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<td>- Controlled release</td>
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<td>- Dispersed reversed microemulsions</td>
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<td>- Solubilization of crystallizing molecules</td>
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<td>- Micellosomes</td>
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2.1. Individual biopolymer molecules

Lipophilic molecules may bind to individual biopolymer molecules at one or more active sites, by either specific or non-specific interactions with different molecular origins. Globular proteins, such as β-lactoglobulin, bovine serum albumin and α-lactalbumin can bind bioactive lipophilic molecules such as resveratrol, docosahexaenoic acid (DHA), conjugated linoleic acid (CLA) and vitamin D to hydrophobic pockets on their surfaces (Wang, Allen, & Swaisgood, 1997; Liang, Tajmir-Riahi, & Subirade, 2008; Zimet et al., 2009). Flexible proteins, such as caseinate, have been shown to bind certain kinds of lipophilic molecules and form molecular complexes that remain dispersed in aqueous solutions (Semo et al., 2007). The driving force for protein binding is usually either hydrophobic or electrostatic in origin. Individual polysaccharide molecules that have ionic or nonpolar side groups may also bind lipids. Starch components (like amylose) and starch derivatives (maltodextrins and cyclodextrins) are able to form helices that have a hydrophobic interior, which are capable of binding non-polar molecules such as fatty acids with appropriate molecular dimensions through hydrophobic interactions (Zabar et al., 2010).

2.2. Single biopolymer molecular clusters

Clusters of biopolymer molecules are also capable of encapsulating certain types of lipophilic molecules. These clusters may be formed from a single type of biopolymer or from mixtures of different types of biopolymers. It was recently shown that casein micelles, which are clusters of casein molecules, are capable of encapsulating and protecting non-polar molecules, such as hydrophobic nutraceuticals, vitamins and drugs (Semo et al., 2007; Shapira, Assaraf, & Livney,
These delivery systems are formed by dissolving the desired lipophilic bioactive in an organic solvent such as ethanol and then adding this solution dropwise into an aqueous solution of sodium caseinate. The casein micelles are then re-assembled by adding calcium, phosphate and citrate to the caseinate solution around neutral pH to promote cluster formation. The encapsulation of vitamin D and DHA in reformed casein micelles and casein nanoparticles showed a remarkable protective effect against degradation, when compared to non-encapsulated ones (Semo et al., 2007; Zimet, Rosenberg, & Livney, 2011).

2.3. Mixed biopolymer molecular clusters

Proteins and ionic polysaccharides form molecular clusters at pH values where there is an electrostatic attraction between them (Matalanis et al., 2011). Recently, Livney and co-workers showed that bioactive lipids (ω-3 fatty acids) can be encapsulated inside molecular complexes of a globular protein (β-lactoglobulin) and an anionic polysaccharide (pectin) (Zimet et al., 2009). Encapsulating these ω-3 fatty acids in biopolymer clusters was shown to improve their oxidative stability. The entrapment by protein, and moreover, the formation of nanocomplexes with the pectin provided good protection against degradation of DHA during an accelerated shelf-life stress test: only about 5–10% was lost during 100 h at 40 °C, compared to about 80% loss when the unprotected DHA was monitored. Similar complexes of β-lactoglobulin and pectin have also been used to encapsulate and protect vitamin D₂ (ergocalciferol). The soluble mixed biopolymer nanocomplexes provided better protection to the vitamin against degradation than single complexes of vitamin D₂ and β-lactoglobulin, and stability was significantly better than that of unprotected vitamin dispersed in water (Ron et al., 2010).
3. Materials and methods

3.1. Materials

Guluronate-rich sodium alginate (ALG, composition: 66.26% (w/w) ALG, 14.19% (w/w) moisture and 9.55% (w/w) ash) from *Laminaria hyperborean* and low viscosity carboxymethyl cellulose (CMC, composition: 92% (w/w) CMC, 7% (w/w) moisture and 1% (w/w) ash) were purchased from BDH Co. (Poole, UK). The molecular weight of sodium alginate was 200 kDa (Smidsrød, 1970) and the uronate compositions were 37.5% manuronate and 62.5% guluronate (Grasdalen, Larsen, & Smidsrød, 1979). κ-carrageenans (KC, 504 kDa, composition: 90% (w/w) KC, 8% (w/w) moisture and 2% (w/w) ash), β-lactoglobulin (BLG, 18.4 kDa, product number L0130, a mixture of genetic variants A and B, composition: 93% (w/w) BLG, 5.4% (w/w) moisture and 1.6% (w/w) ash) from bovine milk, sodium azide (as a preservative), ergocalciferol (vitamin D₂) (VD), β-carotene (βC), curcumin (CUR), folic acid (FA), N-acetyl-L-tryptophanamide and catechin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Deuterium oxide (D₂O, 99.8% D) was obtained from Armar Chemicals (Döttingen, Switzerland). Gum Arabic (GA, 164 kDa, composition: 90% (w/w) GA, 7% (w/w) moisture, 2.5% (w/w) ash and 0.5% (w/w) protein) and immersion oil were purchased from Merck Co. (Darmstadt, Germany). Glacial acetic acid, analytical grade hydrochloric acid, absolute ethanol, 8-anilinonaphthalene-1-sulfonic acid ammonium salt (ANS) and toluene were purchased from Acros Organics (Geel, Belgium). For morphology experiments gum Arabic, sodium alginate (Algogel™) and carrageen (Satiagel™ ME4) purchased from Cargill Co. (Antwerp, Belgium), were used. Moreover, in ligand binding and NMR experiments, BLG isolate (Bipro, minimum purity 90%), obtained from Davisco
Foods International Inc. (Eden Prairie, MN, USA), was utilized. Deionized water (18.2 MΩ cm resistivity) from a Nanopure water system (Nanopure Infinity, Barnstead International, IA, USA) and/or MilliQ water system (Millipore Corporation, MA, USA) was used for the preparation of all solutions. In this study, all materials were used directly from the sample containers without additional purification taking into account their purity.

3.2. Preparation of solutions

ALG, GA, CMC (0.8% (w/w)) and BLG (0.4% (w/w)) stock solutions were prepared by dispersing in deionized water containing 0.03% (w/w) sodium azide. The solutions were then stirred at 250 rpm at ambient temperature for 12 h to ensure complete hydration of the biopolymers in order to use on the following day. KC stock solution (0.5% (w/w)) was prepared by dispersing into deionized water containing 0.03% (w/w) sodium azide at room temperature followed by heating to 85 °C for 30 min under magnetic stirring in order to ensure a complete hydration of polysaccharide.

3.3. Ultrasonic treatment of ALG and KC solutions

ALG and KC stock solutions (30 g) were separately treated by an ultrasonic processor (Hielscher UP200S, power 200 W, frequency 24 kHz, Dr Hielscher Co., Teltow, Germany) for different times (10, 20 or 30 min) at different temperatures (25 or 75 °C) and different amplitudes (50 or 100%). The sample was held in a temperature controlled water bath to prevent the temperature rise by the sonication. A standard tapered horn tip of 5 mm end diameter was immersed 1.5 cm into the
solution during ultrasonication. The ultrasound irradiation was produced directly from
the horn tip under continuous mode.

3.4. Viscosity measurement

3.4.1. Intrinsic viscosity

After ultrasonication, the intrinsic viscosities (IVs) of the control (untreated) and
treated samples of ALG stock solutions were measured at 40 ± 0.1 °C in a capillary
viscometer (Type No. 518 10, Schott Geräte, Hofheim, Germany) using 2 ml of the
sample. Five sodium alginate samples (concentration range from 0.014 to 0.006 %
w/v) were prepared from the stock solution. The uncertainty in flow time was
determined to be 0.04 s. The IVs \([\eta]\) of the samples were obtained by extrapolation
to zero concentration of the experimental dimensionless relative \((\eta_{\text{rel}} = t/t_0)\) and
specific \((\eta_{\text{sp}} = \eta_{\text{rel}} - 1)\) viscosities using Kraemer’s and Huggins’ equations,
respectively

\[
\ln (\eta_{\text{rel}})/C = [\eta] + K''[\eta]^2C \tag{3-1}
\]

\[
\eta_{\text{sp}}/C = [\eta] + K'[\eta]^2C \tag{3-2}
\]

where \(t\) and \(t_0\) are the flow times of the sample and the pure solvent containing
sodium azide, respectively, \(K''\) and \(K'\) are the Kraemer’s and Huggins’ coefficients,
respectively, and \(C\) is the solution concentration. In the current study, the average
value of intercepts obtained from both equations was reported as IV.

3.4.2. Apparent viscosity

The apparent viscosity of the unsonicated (control) and sonicated samples was
measured at 25 °C using a rotational viscometer (Model LV-DVII+, Brookfield
Engineering Laboratories, MA, USA) equipped with spindle number 1 rotated at 10 rpm.

3.5. Turbidimetric analysis at different pHs

The phase diagram during an acid titration was investigated by turbidimetric analysis. Mixtures of BLG + ALG and/or BLG + KC were prepared by first diluting and then mixing the stock solutions at a 2:1 (w/w) protein:polysaccharide mixing ratio and a total biopolymer concentration of 0.15% (w/w). The mixture was acidified gradually by the addition of 0.1 M HCl (pH range of 5-7), 0.4 M HCl (pH range of 3-5) and 2 M HCl (pH range of 1-3) with gentle magnetic stirring for 2 min at each pH level before decreasing it to the next pH. Dilution effects were considered to be minimal. The turbidity (optical density) of the biopolymer mixtures with decreasing pH (from pH ~7 to ~1) was analyzed using a UV/visible light spectrophotometer at 600 nm (BioQuest CE 2502, Cecil Ins., Cambridge, UK) using plastic cuvettes (1 cm path length). Deionized water was used as a blank reference. Critical pH values (pH$_c$: formation of soluble complexes, pH$_{\phi 1}$: formation of insoluble complexes, pH$_{opt}$: maximum optical density, pH$_{\phi 2}$: dissolution of complexes) were measured graphically as the intersection point of two curve tangents according to the methods of Weinbreck, de Vries, Schrooyen, & de Kruif (2003b) and Elmer, Karaca, Low, & Nickerson (2011). BLG, ALG and KC solutions were used as controls at their corresponding concentrations (0.1, 0.05 and 0.05% (w/w), respectively).

3.6. Isothermal titration calorimetry (ITC)

ITC measurements were carried out with a VP-ITC calorimeter (Microcal Inc., Northampton, MA, USA) in order to measure the enthalpic and entropic changes due
to BLG-ALG and BLG-KC interactions at 25 °C. Before titration, the biopolymers were separately dissolved in 5 mM sodium citrate buffer solution (pH 4.25) and stirred for 12 h. The buffer was used to remove the experimental errors resulting from pH mismatch. Heating at 85 °C for 30 min was required for KC. The BLG dispersion containing about 1.5 and 1 mg/ml BLG (for the titration with ALG and KC dispersions, respectively) was filtered through a 0.22-µm low protein binding polyether sulphone (PES) syringe filter (MS® , TX, USA) to obtain aggregate free BLG dispersion. The concentration of BLG dispersion (monomeric equivalent) was measured by UV/visible light spectroscopy using a specific extinction coefficient of 17600 M⁻¹ cm⁻¹ at 278 nm, as reported by Liang et al. (2008) and amounted to 1.013 and 0.828 mg/ml, respectively. The sodium citrate buffer solution was used as blank reference. The dispersions were degassed under vacuum for 6 min by means of a device provided with the ITC apparatus. The injector-stirrer syringe (290 µL) was loaded with ALG and/or KC solutions. Portions of 15 µl (except for the first injection which was 5 µl) of ALG solution (0.1% (w/w) for intact (IN) and sonicated (US) polysaccharides) and/or KC solution (0.1 and 0.175% (w/w) for IN and US polysaccharides, respectively) were injected sequentially into the titration cell (V = 1.405 ml) initially containing either aggregate free BLG dispersion or buffer solution. The duration of each injection was 20 s, and the equilibration time between consecutive injections was 300 s. During the titration, the stirring speed was 310 rpm. The heat of dilution from the blank titration of polysaccharide solution into citrate buffer was measured, and the dilution heat was subtracted from the raw data to measure corrected enthalpy changes. The results are reported as the change in enthalpy per gram of intact and ultrasonicated (for 20 min at 25 °C and amplitude 100%) polysaccharides injected into the reaction cell. The low concentrations of the
biopolymer solutions and the mild temperature supplied a low viscosity at any point of titration, which did not affect the mechanical stirring of the microcalorimeter. Calorimetric data analysis was carried out with Microcal ORIGIN software (v.7.0). Thermodynamic parameters including binding stoichiometry (N), affinity constant (K), enthalpy (ΔH) and entropy (ΔS) changes were calculated by iterative curve fitting of the binding isotherms using the one-binding-site model (assuming the existence of one independent binding site for each protein molecule) provided by the Microcal Origin software and plotted against polysaccharide/protein weight ratio. The Gibbs free energy change (ΔG) was calculated from the equation

\[ ΔG = ΔH - TΔS \]  

(3-3)

3.7. Streaming current detector (SCD)

A Charge Analyser (CA II, Rank Brothers Ltd., Cambridge, England) operating with the streaming current technique was used. A beaker was filled with 70 g of BLG dispersion (0.05% (w/w)) in 5 mM citrate buffer (pH 4.25). The cell was put into the BLG dispersion about 3 mm above the holes in the cylinder and the unit was placed on a stirrer. The stirrer was then started, together with the motor of the SCD. After stabilization of the SCD signal, titration was performed manually using the ALG (IN and/or US) solutions (0.05% (w/w) in 5 mM citrate buffer (pH 4.25) and continued beyond the point where the protein solution was neutralized. The signals were recorded together with the titrant volumes and the zero charge point (ZCP) which is corresponding to maximum neutralization was determined from the titration curve.
3.8. Protein-polysaccharide complexation

Protein-polysaccharide complexes from the mixing of BLG and anionic polysaccharides dispersions at different polysaccharide/protein weight ratios were obtained by the post-blending acidification method. A series of samples containing a fixed protein concentration of 0.1% (w/w) but different polysaccharide concentrations (0–0.2% (w/w)) was prepared by mixing different ratios of BLG and polysaccharide stock dispersions as well as deionized water. Biopolymer solutions were adjusted to pH 4.25 using 0.4, 0.1 and/or 0.01 M HCl solutions. These solutions were stirred for 1 h and then allowed to equilibrate at ambient temperature for 18–24 h prior to analysis.

3.8.1. Turbidity measurement

The turbidity of samples was quantified by their absorbance measured at 600 nm using plastic cuvettes (1 cm path length). Sample solutions were vortexed for 5 s prior to analysis. Highly turbid samples were diluted before measurement using deionized water pre-adjusted with HCl to pH 4.25.

3.8.2. Particle size and zeta- (ζ-) potential analyses

Measurements of particle size distribution were carried out using a dynamic light scattering (DLS) instrument (90Plus, Brookhaven Instruments Corp., Vienna, Austria). Analyses were carried out at a scattering angle of 90° at 25 °C. The effective diameter (also called Z-average mean diameter) was only measured in samples which have shown no sedimentation after equilibration. The Z-average mean diameter was obtained by cumulant analysis. The ζ-potential was determined by laser Doppler anemometry with palladium electrodes using a ZetaPals instrument (Brookhaven Instruments Corp., Vienna, Austria) at fixed light scattering angle of 90°.
at 25 °C. The ζ-potential (mV) was calculated from the electrophoretic mobility using the Helmholtz-Smoluchowski equation. During both dynamic light scattering and electrophoretic light scattering measurements, the viscosity of the continuous phase was assumed to correspond to pure water.

3.8.3. Phase contrast optical microscopy

Protein and anionic polysaccharide complexed mixtures were microscopically characterized at different magnifications using a phase contrast optical microscope (Olympus CX40, Olympus Optical Co., Tokyo, Japan) equipped with a AxioCam ERc 5s video camera (Carl Zeiss Microimaging GmbH, Göttingen, Germany) controlled by an image processor (Kappa ImageBase 2.5). 15 μl of the dispersion was placed between glass slides and then examined. A drop of immersion oil was placed on the glass slide before characterization with 1000 × magnification.

3.9. Bioactives binding to BLG

Fluorescence spectroscopy was used to study the binding of bioactives to BLG by measuring the binding-induced quenching of the intrinsic fluorescence of BLG tryptophanyl residue (Trp19), which is particularly sensitive to changes of its microenvironment (Wang, Allen, & Swaisgood, 1998). The other tryptophan of BLG, (Trp61), is located at the aperture of the barrel and is “silent” in the emission of native BLG because of the proximity of a disulfide moiety (Harvey, Bell, & Brancaleon, 2007). BLG stock solutions were made fresh daily by dissolving in either 10 mM phosphate buffer at pH 7 or 10 mM acetate buffer at pH 4.25 (to determine the effect of pH on the binding of ligands). After filtration through a 0.22-µm syringe filter to obtain aggregate free BLG dispersion, the protein concentrations in the solutions were determined by UV spectroscopy using an Ultrospec 1000 UV-visible
spectrophotometer (Pharmacia-Biotech, Biochrom Ltd., Cambridge, England). The samples were measured in a 1 cm quartz cell at 278 nm against buffer solutions as the reference. BLG concentrations were calculated using an extinction coefficient ($\varepsilon$) of 17600 M$^{-1}$ cm$^{-1}$ (Liang et al., 2008) and amounted to 2.90 and 3.52 µM at pH 7 and 4.25, respectively. The low concentration of the BLG solutions avoided inner filter effects which could occur during the experiment. To facilitate the binding of nutraceutical components to BLG, they were prepared daily by dissolving in absolute ethanol, except with FA which was dissolved in phosphate buffer (pH 7). To prevent degradation, nutraceutical stock solutions of the appropriate concentrations ($\beta$C: 0.130 and 0.104 mM, FA: 0.213 and 0.199 mM, CUR: 0.201 and 0.255 mM, VD: 0.393 and 0.509 mM, at pH 7 and 4.25, respectively) were purged with nitrogen gas, and stored in the dark at 4 ºC. Samples were prepared at room temperature in 2.5 ml plastic tubes covered with aluminum foil, by mixing BLG (2 ml) and different amounts of nutraceutical (0, 2, 5, 9, 14, 20, 27, 35, 44, 54 µl) stock solutions. The protein-nutraceutical solutions were vortexed for 30 s and allowed to equilibrate for 10 min prior to fluorescence measurement. As the ethanol dissipates in the water, most of the nutraceutical components bind to the protein’s binding site(s). The highest resulting ethanol concentration was about 2.7% (v/v), which had no appreciable effect on protein structure. Fluorescence spectra were recorded at room temperature on a Cary Eclipse Fluorescence spectrophotometer (Varian Inc., Walnut Creek, CA, USA) using an excitation wavelength of 287 nm and an emission wavelength of 332 nm (Cogan, Kopelman, Mokady, & Shinitzky, 1976). The band slit (spectral resolution) was 5 nm for both excitation and emission. To eliminate the effects of protein dilution by the added ligand solution and the possible tryptophan fluorescence changes induced by ethanol, for each sample, a blank BLG solution
containing an identical concentration of ethanol (and/or buffer for FA) was prepared and treated in the same manner as the sample. A second blank containing N-acetyl-L-tryptophanamide (NATA) was also prepared in a manner similar to all samples. NATA is unable to interact with nutraceutical models; however it displays a fluorescence spectrum typical of tryptophan. The decrease in fluorescence intensity of blanks containing NATA is not due to the interaction but it results from the inner filter effect as a consequence of ligand absorbance at 287 nm. NATA was also used to exclude the possibility of unspecific interactions of the nutraceutical model with the protein’s tryptophan indoles, where at increasing nutraceutical concentrations the nutraceutical may absorb light, which would otherwise excite the indole groups, and thus fluorescence would decrease for this reason (Dufour, & Haertlé, 1990). The concentration of NATA (0.228 mg/150 ml buffer and 0.265 mg/150 ml buffer, at pH 7 and 4.25, respectively) had been selected in the way that it had the same emission at 332 nm as the studied BLG solution. The fluorescence intensity changes of the blanks were subtracted from fluorescence intensity measurements of the ligand/protein complexes for every considered sample. In these experiments, before correction for the blanks, the fluorescence intensity at 332 nm of the original BLG solution was normalized to 1. All experiments were run in duplicate samples put in quartz cuvettes of 1 cm optical path length. After correcting for the blanks, the differences in fluorescence intensity at 332 nm between complex and free protein were used to measure the apparent dissociation constant ($K'_d$) and the apparent mole ratio of ligand to protein at saturation ($n$). The direct linear plotting method of Eisenthal and Cornish-Bowden (1974), where the corrected fluorescence is plotted directly against ligand concentrations, was used to obtain $K'_d$ directly from the median of intersecting regression lines representing individual observations on the
abscissa. The $n$ values were obtained directly from the fluorescence titration curve plotted against nutraceutical/protein molar ratio correlating to the saturation point.

### 3.10. NMR studies of ligand binding to BLG

Interactions between BLG and some ligands including ANS, toluene, catechin and folic acid were investigated by proton nuclear magnetic resonance (1H NMR) spectroscopy. Stock solutions of ligands of appropriate concentration were prepared by dissolving in deuterium oxide (D$_2$O). To determine the effect of protein on ligand binding, BLG was added to ligand stock solution at different concentrations. All NMR experiments were performed on a BrukerAvance II spectrometer operating at a $^1$H-frequency of 700.13 MHz. A 5 mm TXI gradient probe with a maximum gradient strength of 57.5 G·cm$^{-1}$ was used throughout. Temperature was controlled to within $\pm0.1$ °C with a Eurotherm 2000VT controller. Diffusion coefficients were measured by PFG-NMR with a convection compensated double-stimulated-echo experiment (Jerschow,& Muller, 1997) using monopolar smoothened square shaped gradient pulses and a modified phase cycle (Connell, Bowyer, Bone, Davis, Swanson, Nilsson Morris, 2009).

The echo-decay of the resonance intensity obtained with the double stimulated echo sequence obeys equation 4-5, from which it is clear that the diffusion coefficient $D$ is derived from the echo-decay as a function of the parameter $k$

$$I = I_0 \exp[-D(\gamma G \delta s)^2 \Delta J]$$

$$I = I_0 \exp[-D \cdot k]$$

(3-4)
where \( I \) is the echo intensity with gradient; \( I_0 \) is the echo intensity at zero gradient; \( \gamma \) is gyromagnetic ratio; \( G \) the maximum gradient amplitude; \( \delta \) the duration of the gradient pulse and \( \Delta \) is the diffusion delay corrected for the finite gradient pulse duration \( \Delta = \Delta - 0.6021 \cdot \delta \). The gradient shape factor \( s \) was set to 0.9, to account for the smoothed rectangular gradient shape used in the experiments.

3.11. Nanoencapsulation of nutraceutical models

After finding the appropriate conditions for nanoparticle formation (Section 4.9.) as well as finding nutraceutical binding characteristics to BLG (Section 4.10.), a series of solutions containing constant final BLG concentration (0.1% w/w), and constant final nutraceutical concentration at a molar ratio of 1:1 were prepared by adding nutraceutical compound dissolved in absolute ethanol (except for folic acid which was dissolved in deionized water) to the protein solutions. The protein solution (at neutral pH) was then stirred for half an hour. Polysaccharide stock solution was added to the BLG-nutraceutical solution at the desired quantity (to obtain a transparent system containing nanoparticles) and deionized water was added to obtain a final constant volume and protein concentration. Then the pH was adjusted while stirring to 4.25, and the samples were stirred further for half an hour for equilibration. For each sample, a blank consisting of deionized water which contained an identical concentration of nutraceutical compound dissolved in ethanol (and/or deionized water for folic acid) was prepared and treated in the same manner as the sample. A second blank containing BLG and nutraceutical compound without polysaccharide was also prepared in a manner similar to all samples.
3.12. Statistical analysis

Measurements were performed at least two or three times using freshly prepared samples and analyzed by ANOVA using the MSTATC program (version 2.10, East Lansing, MI, USA). Results were reported as means and standard deviations. Comparison of means was carried out using Duncan’s multiple range tests at a confidence level of 0.05.
4. Results and discussion

4.1. Changes in viscosity after sonication

The effectiveness of the sonication has been evaluated by measuring the changes in apparent and/or intrinsic viscosities which is shown versus sonication time at different amplitudes and temperatures in Fig. 4.1. There was a severe decrease in the viscosity of the KC and ALG solutions. As an example, the viscosities of 19 and 46 mPa.s for the untreated KC and ALG solutions decreased to about 3 and 5 mPa.s after sonication for 30 min at 25 °C and amplitude 100%. This phenomenon is due to the cleavage of the polysaccharide backbone which results in a decrease in the molecular weight of ultrasonically treated polysaccharides and hence decreasing the effective volume of the polysaccharide chains (Weiss, Kristbergsson, & Kjartansson, 2011). The depolymerization process occurs through the effects of acoustic cavitation and can involve two possible mechanisms: mechanical degradation of the polymer from collapsing cavitation bubbles and chemical degradation as a result of the chemical reaction between the polymer and high energy molecules such as hydroxyl radicals produced from cavitation (Chemat, Huma, & Kamran Khan, 2011). According to Iida, Tuziuti, Yasui, Towata, & Kozuka, (2008), the effect of ultrasonication on viscosity depression is extremely dependent on the mechanical and structural properties of the polysaccharides, i.e. whether the polysaccharides have a stiff linear or random coil configuration. For example, pectin showed a rather small change (about 50% decrease) in viscosity, whereas glucomannan showed a much more severe decrease in viscosity by sonication (Iida et al., 2008). The alginate type used in the current study was rich in guluronic acid
residues which are responsible for the stiff nature of the alginate chain (Draget, Moe, Skjåk-Bræk, & Smidsrød, 2006).

Fig. 4.1: Effects of sonication on the apparent and intrinsic viscosity reduction of the KC and ALG stock solutions at different amplitudes and temperatures as a function of sonication time: (♦) Amp. 50%, Temp. 75 °C; (■) Amp. 100%, Temp. 75 °C; (▲) Amp. 50%, Temp. 25 °C; (●) Amp. 100%, Temp. 25 °C. The experiments were made in triplicate. Error bars represent experimental standard deviation.
Fig. 4.1 clearly shows that the sonication temperature had an inverse effect on the viscosity depression when the other parameters (time and amplitude) remained constant. However, this effect was less pronounced at higher sonication times. Iida et al. (2008) also reported that the viscosity depression of a gelatinized starch solution upon sonication was less pronounced at higher sonication temperature. This behaviour may be due to the molecular structure and flexibility which has been shown to be the primary factor that influences the susceptibility to the ultrasonic depolymerization processes (Ram & Kadim, 1970). Increasing the sonication temperature may increase the flexibility of the molecular chain. According to Weiss et al. (2011), flexible biopolymer chains are less susceptible to decreases in viscosity upon ultrasonication. An increase in temperature also leads to an increase in water vapor pressure, which penetrates in larger amounts into the cavitation bubbles and weakens the collapse energy by the so-called “cushioning effect” (Kardos, & Luche, 2001). The viscosity of the KC and ALG solutions decreased significantly (*p<0.05) with increasing time and amplitude of the ultrasonication process, and tended to approach a limiting viscosity value, which may correspond to low molecular weight fractions for which the application of high-intensity ultrasound does not lead to further backbone breakdown (Baxter, Zivanovic, & Weiss, 2005). Similar results have been reported by Grönroos, Pirkonen, & Ruppert (2004) and Wong, Kasapis, & Huang (2012) for carboxymethylcellulose and cellulose depolymerization, respectively. It should be noted that there was a similar pattern for intrinsic viscosity (as a unique function of molecular weight) depression as for apparent viscosity reduction. As an example, the IV of 60 deciliter (dl)/g for the ALG control (untreated) sample decreased to 20 dL/g after sonication for 30 min at 25 °C and amplitude 100%. Differences between data of the control sample obtained in the current study and
those reported in literature (Smidsrød, 1970; Martinsen, Skjåk-Bræk, Smidsrød, Zanetti, & Paoletti, 1991) may be attributed to ALG uronate composition, suppliers and the properties of the solvent (such as pH and ionic strength) in which the biopolymer has been hydrated. In the current study, low ionic strength conditions resulted in an expanded conformation by excluding charge screening effects. Thus, the macromolecular volume in solution was larger, resulting in higher intrinsic viscosity.

4.2. Turbidimetric analysis

Turbidimetric analysis as a function of pH was used to study the kinetics of associative phase separation within mixed BLG-ALG and BLG-KC systems. Indeed, pH affects the ionization degree of the functional groups of the protein and polysaccharide and electrostatic complexing takes place under acidification (Weinbreck et al., 2003). Phase diagrams showing critical pH values of 2:1 protein-polysaccharide mixtures titrated with HCl are shown in Fig. 4.2. A 2:1 ratio of protein to polysaccharide is usually used to investigate the kinetics of the associative phase separation as a function of pH (Schmitt et al., 1999; Hasandokht Firooz, Mohammadifar, Haratian, 2012). In the absence of protein, ALG and KC solutions remained transparent indicating that they did not form particles large enough to scatter light strongly due to electrostatic repulsion between them over nearly the whole pH range considered. However, for ALG, the turbidity increased slightly at lower pH which can be attributed to the fact that the magnitude of the electrical charge on the ALG molecules became relatively small due to protonation of carboxyl groups along the ALG backbone. The sulfate groups of KC were always ionized,
giving the molecules an electrostatic repulsion and hence transparency over the entire pH range.

Fig. 4.2: Phase diagrams showing critical pH values as a function of pH for BLG-KC (a) and BLG-ALG (b) mixtures: (▲) BLG dispersion (0.1% (w/w)), (×) Polysaccharide (IN) and (●) Polysaccharide (US) dispersions (0.05% (w/w)); (■) Protein-Polysaccharide (IN) and (●) Protein-Polysaccharide (US) at 0.15% (w/w) total biopolymer concentration and Protein:Polysaccharide weight ratio of 2:1. (pH_c: formation of soluble complexes, pH_{φ1}: formation of insoluble complexes, pH_{opt}: maximum optical density, pH_{φ2}: dissolution of complexes). The experiments were made in duplicate. Error bars represent experimental standard deviation.
The BLG dispersion showed a broad peak in the measured turbidity versus pH profile with a maximum value around pH 4 to 5 due to self-association around the Ip of BLG, whereas the turbidity decreased as the pH became more acid or alkaline. Similar results were reported by Mounsey et al. (2008). The driving force for protein aggregation around the Ip is probably a combination of hydrophobic attraction, van der Waals attraction and some electrostatic attraction between positive groups on one protein and negative groups on another (Harnsilawat et al., 2006). The pH that resulted in maximum aggregation observed in the BLG dispersion (around the Ip) was significantly (*p<0.05) lower in the presence of ALG, which indicated that the ALG stabilized the proteins against aggregation around the protein’s Ip, presumably by increasing the electrostatic and steric repulsion between the biopolymer particles. Generally, protein-polysaccharide (US) complexed solutions showed lower turbidity than protein-polysaccharide (IN) solutions which can be attributed to the production of smaller polysaccharide chains after sonication. At pH > 5.30, biopolymers were considered to be co-soluble, although a very slight increase in turbidity of the systems can be seen (Fig. 4.2) which may be the result of prevalent non-coulombic interactions such as hydrophobic and hydrogen bindings (Liu, Low, & Nickerson, 2009). Previous researchers have also found little interaction between BLG and pectin at high pH values (Girard et al., 2002a). Another possibility is that weak local electrostatic interactions may occur between protein and polysaccharide molecules as shown in work by Dickinson & Galazka (1991). They have demonstrated that native BLG and anionic polysaccharides (dextran sulfate and propylene glycol alginate) could form ionic complexes at neutral pH due to charge-induced charge interactions. One beneficial consequence of this complexation is the protection against a loss of solubility due to aggregation induced by heating or high-pressure
processing. This can be attributed to stabilization of the native protein structure in the complexed state, and also to blocking of potentially hydrophobic binding sites on the partially unfolded globular protein due to the close proximity of the bulky polysaccharide (Dickinson, 2008). Soluble complexes were formed at a pH$_c$ (~5.30-5.40) that was independent of the polysaccharide type and its treatment (sonicated or non-sonicated). Weinbreck et al. (2004a) reported a pH$_c$ value of 5.5 for different mixtures of whey protein isolate and non-gelling carrageenan (comprised mainly λ-carrageenan). According to Turgeon et al. (2009) and Weinbreck et al. (2004a), this transition occurs at the molecular level (i.e. the complexation begins with a single polysaccharide chain binding to a defined amount of protein). Therefore, it is a local interaction, not influenced by events elsewhere along the polymer chain or events on the other chains, and is therefore not affected by chain length or macromolecular concentrations, but ionic strength (Antonov, Mazzawi, & Dubin, 2010). Formation of soluble complexes occurred at a pH$_c$ above the Ip of the BLG (~4.7-5.2) (Santipanichwong, Suphantharika, Weiss, & McClements, 2008) which is thought to be due to the ability of the globular proteins for charge regulation around the Ip resulting from their electrical capacitance properties (Dickinson, 2008) and/or due to the presence of positive patches (localized regions with higher charge density) on the surface of BLG as a result of low ionic strength conditions which inhibit charge screening (Weinbreck et al., 2003; Turgeon et al., 2009). BLG has several charged patches (basic peptides 1–14, 41–60, 76–83 and 132–148, the latter being part of the α-helix), which are sensitive to complexation with polyanions above the proteins’ Ip (Girard, Turgeon, & Gauthier, 2003a). When the pH decreased further, the critical pH$_{φ1}$ (~3.65-3.80 for ALG and ~4.85 for KC) was reached as a result of nucleation and growth-type kinetics (Sanchez et al., 2006). The pH where there was first an
appreciable increase in turbidity when the pH was reduced was lower for ALG than for KC, which can be attributed to the higher linear charge density of ALG. At this point, more and more protein molecules become attached to the polysaccharide (due to an increase in charge density of the protein) until electroneutrality was attained yielding neutral interpolymeric complexes (Turgeon et al., 2009). Diminishing the net charges on the macromolecular reactants reduces both the hydrophilicity and the solubility of the resultant complex (Tolstoguzov, 1997). This step appears as an intermediate process before the system undergoes extensive higher-order aggregation and bulk phase separation (Laneuville et al., 2005b). As shown in Fig. 4.2.b, pH φ1 is affected slightly by the ALG sonication. The starting pH of the interpolymeric complex formation increases with decreasing charge density of the ALG (US) as discussed later (section 4.5.2.). It should be noted that the measured optical density is the result of the number and size of the biopolymer complexes. The highest amount of BLG-ALG and BLG-KC interactions (pHopt) occurred at pH values of (2.05-2.35) and (1-2), respectively. The peak in the turbidity–pH profile occurred at a pH value lower than the pKₐ of carboxyl groups (~3.5) which may have been because protein-polysaccharide association is the result of a balance of various attractive forces (e.g. van der Waals, hydrophobic, and electrostatic interactions between oppositely charged groups) and various repulsive forces (e.g. electrostatic interactions between similarly charged groups). Consequently, the maximum amount of aggregation occurred when the protein has a very high net charge and polysaccharide starts to aggregate due to low charge. A single protein molecule may be able to bind to more than one polysaccharide chain, so that the proteins can act as electrostatic bridges (Jones, Lesmes, Dubin, & McClements, 2010b). At pH < pHopt, the turbidity of the BLG-ALG complexes was reduced as a result of the
extensive protonation of the alginate carboxyl groups. There was a little difference in pH$_{φ2}$ (1.20 and 1.40) for ALG (IN)- and ALG (US)- BLG complexed systems, respectively. However, the turbidity of the complexes was significantly (*$p<$0.05) higher as compared to BLG and ALG alone, which may be due to bridging flocculation which led to obvious precipitation after storage at pH < pH$_{φ2}$. In this study, pH$_{φ2}$ was absent for BLG-KC mixtures, since the dissociation of KC’s sulphate groups is not suppressed at low pH and they remain charged (Turgeon et al., 2009). It is worth mentioning that some authors have found other critical pH values, notably for protein conformational changes following binding or for morphological changes in the coacervates droplets in the β-lactoglobulin–acacia gum system using a multi-methodological approach (Mekhloufi et al., 2005). In subsequent experiments the target pH was chosen to be 4.25, based on the pH of a clear traditional herbal beverage in order to assess the capability of the produced nanoparticles as delivery systems for fortification purposes in the future.

4.3. ITC results

ITC is a powerful technique to determine the type and magnitude of the energies involved in the complexation process of biopolymers by titrating one binding partner with another while measuring the heat released in a calorimeter cell. It has been successfully applied in recent years in a growing number of studies of protein-polysaccharide complexes and coacervates (Girard et al., 2003b; Schmitt et al., 2005; Harnsilawat et al., 2006; Guzey et al., 2006; de Souza, Bai, Gonçalves, & Bastos, 2009; Aberkane et al., 2010). The heat flow versus time profiles resulting from the titration of BLG with intact and sonicated polysaccharides at 25 °C and pH 4.25 are shown in Fig. 4.3.a-d, respectively.
Fig. 4.3: a and b: Thermograms corresponding to the titration of the BLG dispersion (0.1013% w/v) with ALG (IN) and ALG (US) dispersions (0.1% w/w), respectively in 5 mM sodium citrate buffer (pH 4.25) at 25 °C; c and d: Thermograms corresponding to the titration of the BLG dispersion (0.0828% w/v) with KC (IN) and KC (US) dispersions (0.1% and 0.175% w/w, respectively) in 5 mM sodium citrate buffer (pH 4.25) at 25 °C.

The area under each peak represents the heat exchange within the cell containing BLG after each polysaccharide injection. The injection profiles in the sample cell were exothermic and decreased regularly to a state of thermodynamic stability (about zero) after the 11th and 18th injection of ALG (IN) (0.1% w/w) and ALG (US) (0.1% w/w), and after the 15th and 12th injection of KC (IN) (0.1% w/w) and KC (US) (0.175% w/w), respectively. Exothermicity is associated with the nonspecific electrostatic neutralization of the opposite charges carried by the two biopolymers.
indicating an enthalpic contribution of complex coacervation (Girard et al., 2003b; Schmitt et al., 2005), while its regular decrease is attributed to a reduction in free protein remaining in the reaction cell after successive injections, which explains the lowering of the energy released. Girard et al. (2003b) reported a similar exothermic sequence for BLG interaction with low- and high-methoxyl pectin, while Aberkane et al. (2010) and Nigen et al. (2007) reported an exothermic-endothermic sequence as indicative for the other energetic contributions such as the liberation of water molecules and ions, conformational changes of biopolymers, and/or the aggregation of protein-polysaccharide complexes. To characterize thermodynamic parameters, the binding isotherms obtained by integrating of the isotherm peaks and subtraction of the heats of dilution of polysaccharides into buffer solution were fitted using the one site binding model provided by the Microcal Origin software and plotted against polysaccharide/protein weight ratio (Fig. 4.4.a and b). The first injection was not taken into account for analysis. The calculation gives a typical sigmoidal saturation curve, which can be considered as a progressive binding of the BLG molecules present in the titration cell to the binding sites along the polysaccharide backbone. The isoenthalpic plateau observed in the binding isotherms was reached at ALG (IN) and ALG (US) to BLG weight ratios of about 0.14 and 0.19, and at KC (IN) and KC (US) to BLG weight ratios of about 0.20 and 0.30, respectively. The obtained results for native ALG are in reasonable agreement with the ones obtained by Harnsilawat et al. (2006) for ALG-BLG interactions at pH 4. However, they reported a small endothermic peak at 0.41 µM sodium alginate (ALG (IN)/BLG weight ratio 0.0874) after a relatively high exothermic enthalpy change, followed by a fairly small exothermic enthalpy change at higher sodium alginate concentrations, which may be
due to some different kinds of binding mechanism, or some rearrangement of the proteins and polysaccharides within the complexes formed (Harnsilawat et al., 2006).

**Fig. 4.4:** (a) Binding isotherms corresponding to the titration of the BLG dispersion (0.0828% w/v) with (■) KC (IN) and (●) KC (US) dispersions (0.1% and 0.175% w/w, respectively) in 5 mM sodium citrate buffer. (b) Binding isotherms corresponding to the titration of the BLG dispersion (0.1013% w/v) with (■) ALG (IN) and (●) ALG (US) dispersions (0.1% w/w) in 5 mM sodium citrate buffer (pH 4.25) at 25 °C as a function of ALG/BLG weight ratio.

To the best of our knowledge, the interaction between KC and BLG has not been studied before using ITC. Although the titration of the sonicated polysaccharides into
the BLG dispersion had a similar ITC pattern to those of the intact polysaccharides, it
should be noted that the interaction was less exothermic (~2 times) for ALG (US)-
BLG interaction and/or occurred with significantly (*p<0.05) lower affinity constant in
the case of KC (US)-BLG interaction (Table 4.1). This observed difference may be
explained from the fact that the negative charge densities on the sonicated
polysaccharide molecules is smaller than those of intact polysaccharides as
discussed later (section 4.5.2.), which resulted in a decrease in the strength of the
interaction and/or affinity constant. The decrease in the affinity constant of the KC
(US)-BLG interaction can also be attributed to the changes in the helical structure of
the polysaccharide after sonication. Thermodynamic parameters including binding
stoichiometry (N), affinity constant (K), enthalpy (ΔH) and entropy (TΔS)
contributions and Gibbs free energy change (ΔG) found for the interaction between
polysaccharides and BLG are shown in Table 4.1.

<table>
<thead>
<tr>
<th>Complex</th>
<th>N (mg PS/g BLG)</th>
<th>K (g⁻¹.l)</th>
<th>ΔH (cal.g⁻¹)</th>
<th>TΔS (cal.g⁻¹)</th>
<th>ΔG (cal.g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALG (IN)-BLG</td>
<td>92.7 ± 0.8</td>
<td>1230 ± 210</td>
<td>-4.014 ± 0.052</td>
<td>-3.950 ± 0.031</td>
<td>-0.064 ± 0.021</td>
</tr>
<tr>
<td>ALG (US)-BLG</td>
<td>172.6 ± 1.7</td>
<td>900 ± 208</td>
<td>-2.158 ± 0.033</td>
<td>-1.950 ± 0.047</td>
<td>-0.208 ± 0.055</td>
</tr>
<tr>
<td>KC (IN)-BLG</td>
<td>192.3 ± 1.4</td>
<td>10476 ± 6032</td>
<td>-2.706 ± 0.042</td>
<td>-2.680 ± 0.034</td>
<td>-0.026 ± 0.008</td>
</tr>
<tr>
<td>KC (US)-BLG</td>
<td>214.1 ± 3.0</td>
<td>535 ± 137</td>
<td>-2.940 ± 0.062</td>
<td>-2.780 ± 0.038</td>
<td>-0.160 ± 0.024</td>
</tr>
</tbody>
</table>

The binding enthalpy was negative and favorable, whereas the binding entropy was
unfavorable (negative) during polysaccharide-BLG interaction. According to Ou et al.
(2006) the complexation between weakly charged polyelectrolytes is driven by a
negative enthalpy due to the electrostatic interaction between two oppositely
charged components, while counterion release entropy plays only a minor role. The
unfavorable entropic effects originate mainly from the loss in biopolymer conformational freedom after association (Dickinson, 2008). BLG and intact polysaccharides interacted with a high affinity constant and a strong enthalpy change. Using ITC we have calculated, apparently for the first time, that about 117 and 142 BLG molecules were involved in the interaction process with intact ALG and KC, respectively, by assuming molecular weights of 200 kDa for ALG (IN) and 504 kDa for KC (IN). Schmitt et al. (2005) and Aberkane et al. (2010) reported values for the enthalpy change ((-0.933 ± 0.001) and (-1.072 ± 0.014) cal.g⁻¹), affinity constant ((25.4 ± 13.0) and (896 ± 66) g⁻¹.l) and binding stoichiometry (341.3 and 357.2 mg GA/g BLG) upon complexation of BLG with Acacia gum (MW ~ 540 kDa) at pH 4.2, respectively. The differences can be explained by the higher charge densities on the intact ALG and KC molecules than on Acacia gum molecules. The interaction between BLG and sonicated polysaccharides occurred with higher binding stoichiometry values. The fact that the minimum required amount of the sonicated polysaccharide to interact with all of the BLG molecules present in the reaction cell was higher than that of intact polysaccharide suggests that the ratio of the protein bound per gram of polysaccharide was greater for intact polysaccharide than sonicated one. Therefore, there are more binding sites available on intact polysaccharide for the positively charged protein molecules. A possible explanation for the reduction in the number of binding sites is that the sonication process promoted polysaccharide-polysaccharide interactions. Hence, saturation (isoenthalpic plateau) occurred at a higher polysaccharide to protein weight ratio. These results are in good agreement with those of Sperber et al. (2009) and Weinbreck et al. (2004a). They found that BLG binds to the anionic polysaccharides (pectin and carrageenan) more strongly when their charge density increased, and
that the number of protein molecules bound per polysaccharide chain increased. These results are also in good agreement with those of Chang, McLandsborough and McClements (2012). They found that $\varepsilon$-polylysine (an antimicrobial cationic polyelectrolyte) interacted with an anionic polysaccharide (pectin) more strongly when the charge density on the pectin molecules increased (i.e. with decreasing degree of esterification). The unfavorable entropic contribution ($T\Delta S$) was relatively in the same range as the favorable enthalpic contribution, indicating that any change in enthalpy is accompanied by a similar change in entropy, that is, entropy-enthalpy compensation occurred (Aberkane, Jasiewski, Gaiani, Hussain, Scher, & Sanchez, 2012). The changes in Gibbs free energy were negative for all polysaccharides indicating the spontaneous nature of the interactions. The difference in Gibbs free energy changes between intact and sonicated polysaccharides of the same type can be attributed to the fact that the loss in polysaccharide conformational freedom after association is more considerable for larger molecules than smaller ones. Finally, it is difficult to assign a precise molecular change in the system to the observed enthalpy change because the overall measured signal is a combination of enthalpy changes associated with different molecular phenomena such as conformational changes, various kinds of association-dissociation processes and counter ion binding-dissociation (Harnsilawat et al., 2006; Guzey et al., 2006).

4.4. SCD results

The SCD technique can be used to measure the charge density of dissolved and colloidally dispersed polyelectrolytes (Xu & Deng, 2003). The SCD signal is related to the electrokinetic charge of colloidal materials in the sample (Tan, Koopal, Weng, van Riemsdijk, & Norde, 2008). In this study, the maximum of the BLG
neutralization was investigated by studying their interactions with the ALG solutions. This corresponds to the point where the streaming current is around zero (Kam & Gregory, 1999). The SCD signal versus ALG/BLG weight ratio profile is shown in Fig. 4.5. Generally, the shapes of the SCD curves for both types of ALGs were similar, with a fairly sharp break from positive to negative streaming current values. The ZCP was attained sooner during titration with ALG (IN) solution which is in reasonable agreement with the results of the ITC (Fig. 4.4). In addition, the values of ZCPs are in reasonable agreement with the maximum of the turbidity versus ALG/BLG weight ratio profiles (Fig. 4.6.a and 4.7).

**Fig. 4.5:** Streaming current curves corresponding to the titration of the BLG dispersion (0.05\% w/w) with (□) ALG (IN) and (●) ALG (US) dispersions (0.05\% w/w) in 5 mM sodium citrate buffer (pH 4.25) at 25 °C as a function of ALG/BLG weight ratio.
4.5. Complex evaluation

4.5.1. Turbidity versus polysaccharide/protein weight ratio profiles

The turbidity (absorbance or optical density) of the protein-polysaccharide mixed solutions was measured as a function of polysaccharide/proteinweight ratio at pH 4.25 to provide some deeper insights into the mechanisms of complexed biopolymer nanoparticle formation (Fig. 4.6.a-d) and also to find the most suitable conditions for forming stable nanoparticles. The initial turbidity of the BLG suspension in the absence of polysaccharide was about 0.113, because of some aggregation of proteins around pH 4.25. The results clearly showed that polysaccharide type had a major impact on the electrostatic complexation of BLG. The differences between polysaccharides can be interpreted in terms of differences in their molecular structure, the nature of the complexes formed, and the strength of the electrostatic interactions between the protein and polysaccharide molecules. ALG, KC and GA behaved fairly similarly with one peak being decreased by increasing polysaccharide/proteinweight ratio. On the other hand, CMC exhibited a quite different behavior with two peaks in turbidity. This effect might be due to the fact that CMC had higher chain stiffness than the other polysaccharides, which may have limited the formation of the core-shell structure at low polysaccharide/proteinweight ratios. The polysaccharide to proteinweight ratio had a major effect on the solution turbidity and degree of sediment formation in the solutions. For all four intact polysaccharides at low polysaccharide/proteinweight ratios (lower than 0.20, 0.50, 0.50 and 1.00 for ALG, KC, CMC and GA, respectively) complexed biopolymer particles were unstable to aggregation and sedimentation because they can achieve electrical neutrality (protein depletion) due to the high protein binding (Weinbreck et al., 2003b) leading to high turbidity and aggregation as
Fig. 4.6: Optical density profiles of the BLG dispersion (0.1% w/w) mixed with (a): (■) ALG (IN) and (●) ALG (US), (b): (■) KC (IN) and (●) KC (US), (c): (■) CMC and (d): (■) GA dispersions, then acidified to pH 4.25 at 25 °C as a function of polysaccharide/protein weight ratio (total biopolymer concentration range from 0.1% to 0.3% w/w). The experiments were made in triplicate. Error bars represent experimental standard deviation.
seen in Fig. 4.7 (white sediment at the bottom of the glass vials with a clear serum layer on top). When the tubes were shaken, they formed a cloudy homogeneous suspension, which settled out again after storage. Protein-polysaccharide mixtures at different polysaccharide/protein weight ratios leading to precipitation were microscopically characterized just after acidification to pH 4.25, during precipitation and after precipitation (lower phase) (Fig. 4.8). It should be mentioned that phase contrast imaging improves contrast in unstained biological samples by changing the phase of the scattered light and converting refractive index differences to light and dark image regions (Cooper, Dubin, Kayitmazer, & Turksen, 2005). The initial structures are of spherical shape. It seems that complex coacervation in mixed protein-polysaccharide dispersions is a nucleation and growth mechanism. Similar mechanism was reported by Sanchez et al. (2006), in a mixture of BLG with gum Arabic. According to Sanchez et al. (2006), the nucleation and growth mechanism is the general mechanism of complexation/coacervation between biological macromolecules. Complexes grew in size during precipitation and their number was reduced (Fig. 4.8). This feature could be due to coalescence of complexes or Ostwald ripening (Sanchez et al., 2006). These samples are unsuitable for utilization as stable colloidal delivery systems in the food industry. Particles formed in the BLG/KC (US) and BLG/ALG (US) mixed systems did not markedly differ in structure as compared to the previous ones (data not shown). At higher polysaccharide/protein weight ratios the samples were less turbid and did not exhibit sedimentation, indicating that colloidal dispersions containing small stable nanocomplexes with higher stability than the protein aggregates themselves were formed, presumably because the electrostatic and steric repulsion resulting from the presence of a polysaccharide shell around the protein core is sufficiently strong to pr-
Fig. 4.7: Optical images of the BLG dispersion (0.1% w/w) mixed with various polysaccharide dispersions, then acidified to pH 4.25 at different polysaccharide/protein weight ratios (total biopolymer concentration range from 0.1% to 0.3% w/w).
Fig. 4.8: Phase contrast optical micrographs of various polysaccharide-protein mixtures at different weight ratios just after mixing and acidification to pH 4.25, during precipitation and after precipitation (bottom phase).

-event aggregation as revealed by the influence of polysaccharide on the $\zeta$-potential of the complexes (Fig. 4.9). Since the resolution of the phase contrast microscope is not enough to visualize nanoparticles, no structure was detected at higher
polysaccharide/protein weight ratios (Fig. 4.8). Visual appearance is one of the most important attributes for a food product, so these stable colloidal dispersions may have important implications for practical utilization within transparent foods. The transparent samples were obtained at lower ALG/BLG weight ratios as compared to those of the other polysaccharides. This suggested that ALG was more effective at inhibiting protein aggregation than the other polysaccharides, possibly because of its higher charge density. The turbidity profiles of BLG-KC (US) and BLG-ALG (US) were similar to that of BLG-KC (IN) and BLG-ALG (IN). Generally, the lower charge density of sonicated polysaccharide may account for the observed differences in the protein-sonicated polysaccharide turbidity profile including a slight shift to the right, a slight decrease in the maximum turbidity and an increase in the turbidity of the cloudy samples (e.g. the sample representing KC (US)/BLG weight ratio of 0.5) (Fig. 4.6.a, b; Fig. 4.7). The data are consistent with the ITC data, which also indicated that there was a strong interaction. If we compare the turbidimetric results with the ones obtained by calorimetry, we can say that in terms of interpolymeric complex formation, higher turbidity values were observed at higher enthalpy change values. However, the calorimetry showed to be more distinguishable, as a result of its higher sensitivity. Reduction in turbidity (corresponding to the formation of soluble complexes) correlates with the saturation ratio determined from ITC binding curves, where the released energy reached zero.

4.5.2. ζ-potential versus polysaccharide/protein weight ratio profiles

For any delivery system, it is essential that the system remains stable throughout the entire life cycle of the product. Furthermore, the biopolymer nanoparticles should not adversely impact the normal shelf-life of the product itself.
(Matalanis et al., 2011). Electrophoretic mobility (EM) can be obtained from the perturbations of Brownian diffusivity under a pulsating electrical field and is a crucial parameter for predicting the stability of colloidal delivery systems (Cooper et al., 2005; Matalanis et al., 2011). Colloidal particles with ζ-potentials more positive than +30 mV or more negative than -30 mV are normally considered stable (Mounsey et al., 2008). The ζ-potential profiles of the protein-polysaccharide complexed systems as a function of polysaccharide/protein weight ratio at pH 4.25 are shown in Fig. 4.9.
Fig. 4.9: Zeta-potential profiles of the BLG dispersion (0.1% w/w) mixed with (a): (■) ALG (IN) and (●) ALG (US), (b): (■) KC (IN) and (●) KC (US), (c): (■) GA and (d): (■) CMC dispersions, then acidified to pH 4.25 at 25 °C as a function of polysaccharide/protein weight ratio (total biopolymer concentration range from 0.1% to 0.3% w/w). The experiments were made in triplicate. Error bars represent experimental standard deviation.

In the absence of polysaccharide, the ζ-potential of the BLG suspension was around +14 mV, which was due to the fact that BLG was below its Ip and therefore had a net positive charge. The absolute value of the ζ-potential depended considerably on the polysaccharide type (ALG>KC>CMC>GA) and concentration. This effect might be due to the fact that the ALG had a higher negative charge
density than the other polysaccharides at pH 4.25. As the polysaccharide/protein weight ratio increased (from 0 to 0.15 for ALG (IN), 0 to 0.37 for KC (IN), 0 to 0.2 for CMC and 0 to 0.5 for GA) the EM values decreased from positive to negative and the Smoluchowski model yielded ζ-potential values indicating low stability systems which resulted in precipitation. According to Aberkane et al. (2010), the requirement of neutrality at phase separation is not a general rule for protein-polyanionic polymers and phase separation may occur with a negative total charge. Beyond these points, the ζ-potential values remained rather constant at limiting values reflecting an excess of polysaccharide. These measurements showed that negatively charged polysaccharide molecules associated with the surfaces of the positively charged BLG aggregates and caused charge reversal. The ζ-potential profile of the protein-sonicated polysaccharide mixed systems showed a similar trend with lower intensity. Therefore, they were more prone to aggregation than those containing intact polysaccharide particularly at intermediate polysaccharide/protein weight ratios. This trend is in agreement with the charge densities of the two types of ALGs and KCs which may be deduced from the zeta-potential values, which were -60±2, -49±2, -54±5 and -42±4 mV for ALG (IN), ALG (US), KC (IN) and KC (US), respectively, at a concentration of 0.1% w/w and pH 4.25. Tang, Huang, & Lim (2003) reported that the ζ-potential values of chitosan nanoparticles decreased from (47.5±1.3) to (45.5±0.3) after 10 min sonication at amplitude 80% (more gentle conditions than those of the current work). This phenomenon can be attributed to the reduction of the polysaccharide reactivity after sonication, which may be due to some heterogeneous sonochemical interactions and structural changes that took place during the sonication process. Polysaccharide reactivity is governed by the distribution and number of functional groups attached to the polymerized sugar units
that form the backbone of the polysaccharide (Weiss et al., 2011). Polysaccharides subjected to high-intensity ultrasound can undergo a large number of sonochemical reactions including glycosylation, acetalization, oxidation, C-heteroatom, and C–C bond formations (Kardos et al., 2001), which may eliminate the reactive sites present along the polysaccharide backbone or may promote polysaccharide-polysaccharide interactions which reduce the number of binding sites for the BLG molecules resulting in affinity constant and/or interaction strength reduction.

4.5.3. Particle size versus polysaccharide/protein weight ratio profiles

The influence of polysaccharide type and its treatment on the mean hydrodynamic nanoparticle diameter in mixed BLG and anionic polysaccharide solutions showing no precipitation as a function of the polysaccharide/protein weight ratio at pH 4.25 is shown in Fig. 4.10.
Fig. 4.10: Effective diameter profiles of the BLG dispersion (0.1% w/w) mixed with (a): (■) ALG (IN) and (○) ALG (US), (b): (■) KC (IN) and (○) KC (US), (c): (■) CMC and (d): (■) GA dispersions, then acidified to pH 4.25 at 25 °C as a function of polysaccharide/protein weight ratio.

BLG mixtures complexed with ALG, KC and GA behaved fairly similarly with one minimum, while those complexed with CMC exhibited a different behavior with a steady increase in particle size with increasing polysaccharide/protein weight ratio. The complexes were relatively large at low polysaccharide/protein weight ratios (of 0.25 for ALG (IN)/BLG, 0.50 for KC (IN)/BLG and 1 for GA/BLG mixtures) possibly resulting from weak electrostatic repulsion between biopolymer particles (Fig. 4.9) and/or the formation of polymer bridges between particles due to sharing of polysaccharide molecules between protein aggregates at low polysaccharide concentration. The smallest nanoparticles were obtained at ALG (IN)/BLG, KC
(IN)/BLG, GA/BLG and CMC/BLG weight ratios of 0.375, 0.75, 1.5 and 0.375, presenting a mean diameter of 295, 408, 329 and 657 nm, respectively. These complexes showed a lower PDI than the biopolymers themselves. Generally, the shrinkage of the protein-polysaccharide complexes (except for BLG-CMC ones) occurring at low ionic strength could be understood as a reduction of the intramolecular repulsion induced by the interaction of the BLG with the acidic functional (sulphate and/or carboxyl) groups of the polysaccharides. In other words, the neutralization of charges of anionic polysaccharide can also reduce the rigidity of backbone chains due to a decrease in repulsive interactions of like-charged groups (Tolstoguzov, 2003). This compaction phenomenon was well predicted by Monte Carlo simulations which showed that at low ionic strengths, a polyelectrolyte chain would wrap around an oppositely charged spherical particle (Girard et al., 2003b). Indeed, mutual neutralization decreases the net charge, hydrophilicity and chain rigidity of the junction zones resulting in a compact conformation of the complex with the hidden junction zones (Fig. 4.11 and 4.12) (Tolstoguzov, 2003). There was an appreciable increase in the diameter of the particles in the mixed system when the polysaccharide/protein weight ratio was increased. Zimet et al. (2009) concluded that an increase in the polysaccharide concentration increases the viscosity, thereby slowing down the mobility of any particles present (lower fluctuations), which is interpreted by the DLS as an apparently increased particle size. This conclusion is in good agreement with our results since for complexed solutions containing CMC, the increase in particle size was found to be more dependent on polysaccharide concentration due to its ability to form more viscous systems. This conclusion is also in good agreement with our results for complexed solutions containing sonicated polysaccharides of the same type, due to its inability to form viscous systems. Anoth-
Fig. 4.11: Mutual neutralization increases the chain flexibility (Tolstoguzov, 2003).

Fig. 4.12: A highly schematic representation of the compaction phenomenon due to the wrapping process which results in the hidden junction zones.

-er possibility is that the changes in intramolecular repulsion and conformation (adoption of a more extended structure) resulting from the decreased ratio of protein molecules per polysaccharide linear chain (more negative complexes) as well as
polysaccharide stiffness may cause a bigger particle size which is more pronounced for inflexible and linear polysaccharides such as CMC (a biopolymer with highly open structures and an excellent thickening agent) compared to flexible, compact and highly branched ones like GA. Owing to topological limitations, protein globules and rigid anionic polysaccharide chains cannot achieve contact between all their charged groups. On the contrary, flexible polysaccharides tend to form a maximum number of contacts with an oppositely charged protein (Tolstoguzov, 2003). Nonspherical particles have a greater effective volume than an equivalent mass of spherical particles.

The mean diameters of the sonicated polysaccharide-protein nanoparticles were significantly (*p<0.05) smaller than the intact polysaccharide-protein nanoparticles at polysaccharide/protein weight ratios which corresponded to sufficient repulsion between complexed particles. In the presence of sonicated polysaccharide, the effective diameters of the biopolymer complexes remained relatively constant as compared to those containing intact polysaccharide. This may be due to the larger flexibility of the sonicated polysaccharide chains with the reduction of the charge as well as to the lower viscosity of the biopolymer mixtures (Fig. 4.13). Generally, the polydispersity index of the BLG-KC (IN) and BLG-ALG (IN) nanoparticles (0.313 and 0.296, respectively) at weight ratio (0.75 and 0.375, respectively) corresponding to minimum particle size was significantly (*p<0.05) higher than that of BLG-KC (US) and BLG-ALG (US) nanoparticles (0.151 and 0.258, respectively) at weight ratio of 1 (minimum particle size), indicating the consequence of polysaccharide sonication was the homogenization of particle sizes in the mixed dispersion. One should keep in mind that the measured results are intensity-weighted, which means that the larger particles have the larger contribution.
If volume- or number- weighted distributions are considered, much smaller average diameters are obtained.

**Fig. 4.13:** Translational diffusion coefficient of BLG-ALG mixtures as affected by polysaccharide sonication

### 4.6. Binding properties of the nutraceutical compounds to BLG

BLG as a small globular protein contains 162 amino acid residues with one free thiol group and two disulfide bonds and has a molecular weight of 18.4 kDa (Fox, 2009). It is a member of the lipocalin family of proteins because of its ability to bind small hydrophobic molecules into a hydrophobic cavity (Kontopidis, Holt, & Sawyer, 2004). The most abundant variants of bovine BLG are BLG A and BLG B which differ by two amino acid residues, Asp64Gly and Val118Ala, respectively (Farrell et al., 2004). The quaternary structure (association properties) of the protein varies among monomers, dimers or oligomers depending on the pH, temperature, concentration and ionic strength as a result of a delicate balance among hydrophobic, electrostatic and hydrogen-bond interactions (Sakurai, & Goto, 2002; Gottschalk, Nilsson, Roos, & Halle, 2003). At pH 5–8, BLG exists as a dimer, at pH 3–5 the dimers associate to form octamers, and at extreme pH values (<2 or >8) most protein exists as monomers. At pH>9, the molecule is irreversibly denatured. BLG folds up (Fig. 4.14) into an 8-stranded (A-H), antiparallel β-barrel with a 3-turn α-helix on the outer
surface and a ninth β-strand (I) flanking the first strand. It is this strand that forms a significant part of the dimer interface in the bovine protein.

**Fig. 4.14:** 3D illustration of BLG showing the binding of a ligand (cholesterol) to the calyx, reproduced from Kontopidis et al. (2004).

The so-called calyx, or β-barrel, is conical and is made of β-strands A-D forming one sheet, and strands E-H forming a second. Strand A bends through a right angle such that the C-terminal end forms an antiparallel strand with H; strands D and E also form a less significant interaction completely closing the calyx (Kontopidis et al., 2004). It is this central cavity, the calyx, which provides the main ligand-binding site (Kontopidis et al., 2004; Edwards, Creamer, & Jameson, 2009). On the outer surface of the β-barrel, between strands G and H, is the 3-turn α-helix. The loops that connect the β-strands at the closed end of the calyx, BC, DE, and FG are generally quite short, whereas those at the open end are significantly longer and more flexible. In particular, the EF loop (residues 85-90) acts as a gate over the binding site. At low pH, it is in the “closed” position, and binding is inhibited or impossible, whereas at high pH it is open, allowing ligands to penetrate into the main hydrophobic binding site (Kontopidis et al., 2004). The “latch” for this gate is the side chain of the Glu89 (Qin, Bewley, Creamer, Baker, Baker, & Jameson, 1998).
Ragona, Fogolari, Catalano, Ugolini, Zetta, and Molinari (2003) suggested that the pH-dependent conformational change of the EF loop triggered by the protonation of Glu89 is common to all BLGs and that ligand binding (of palmitic acid) is determined by the opening of this loop. Konuma, Sakurai, and Goto, (2007) suggested that the plasticity of the D strand and the EF and GH loops allows BLG to accommodate a wide range of ligands. Computational studies have shown that three potential binding sites are possible for ligand binding to protein: the canonical site inside the calyx, the surface hydrophobic pocket in a groove between the α-helix and the β-barrel and the outer surface near Trp19-Arg124 (Liang et al., 2008). The central, or main, ligand-binding site seems to accommodate linear molecules like fatty acids and also retinol with the cyclohexenyl ring system inside. The lining of the pocket is very hydrophobic (Kontopidis et al., 2004).

Measurements were made based on the binding-induced quenching of the intrinsic fluorescence of tryptophanyl residue (TRP19) found at the bottom of the calyx (Kontopidis et al., 2004). In this experiment, the corrections for the blanks are of importance. At increasing nutraceutical concentrations, the nutraceutical may absorb light, which would otherwise excite the indole groups, and thus fluorescence would decrease for this reason (Dufour et al., 1990) not for binding. The decrease in the fluorescence intensity of the blanks containing folic acid was higher than that of the blanks containing the other nutraceutical model compounds which can be attributed to the higher UV light absorbance by folic acid (data not shown). The raw data was analyzed to measure the apparent dissociation constant ($K'_d$) and the apparent mole ratio of ligand to protein at saturation ($n$). Firstly, the fluorescence intensity values of the BLG-nutraceutical dispersions (A), experimentally determined for each sample, were normalized after dividing by the fluorescence intensity value
of the original BLG dispersion (B), (A/B). The fluorescence intensity values of the blanks (C) were also normalized in a similar manner after dividing by the fluorescence intensity value of the blank containing no nutraceutical compounds (D), (C/D). The fluorescence intensity changes of the blanks were determined by subtracting the normalized fluorescence intensity value of each blank from 1 (the normalized fluorescence intensity value of the blank containing no ligand), (1-(C/D)). In order to obtain corrected data, the values of (1-(C/D)) were subtracted from the normalized fluorescence intensity values (A/B) of the ligand-protein complexes for every considered sample, ((A/B)-(1-(C/D)) = Y). The fluorescence intensity upon saturation of BLG molecules was visually determined. A typical fluorescence titration curve following BLG’s tryptophan quenching (corrected for the blanks) as a function of nutraceutical/BLG molar ratio is shown in Fig. 4.15.

![Fluorescence Titration Curve](image)

**Fig. 4.15:** A typical fluorescence titration curve of (3.52 and 2.90 μM) BLG dispersions complexed with (0.509 and 0.393 mM) ergocalciferol following BLG’s tryptophan quenching (corrected for the blanks) as a function of ergocalciferol/BLG molar ratio at pH 4.25 (■) and pH 7 (●), respectively.
After correcting for the blanks, the direct linear plotting method of Eisenthal and Cornish-Bowden (1974), where the corrected fluorescence ($Y$) is plotted directly against ligand concentrations ($X$), was used to obtain $K'_d$ directly from the median of intersecting regression lines (drawn between $(-X_i,0)$ and $(0,Y_i)$) representing individual observations $(X_i,Y_i)$ on the abscissa (X axis). The $n$ values were obtained directly from the fluorescence titration curve plotted against nutraceutical/protein molar ratio correlating to the saturation point. The apparent dissociation constant ($K'_d$) and the binding sites per monomer ($n$) are presented in Tables 4.2. and 4.3., respectively.

**Table 4.2:** Apparent dissociation constant ($K'_d$, expressed in nM) of BLG and nutraceutical model compounds at pH 7.00 and 4.25.

<table>
<thead>
<tr>
<th>pH</th>
<th>$K'_d$(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$-carotene</td>
</tr>
<tr>
<td>7.00</td>
<td>$21 \pm 3^B$</td>
</tr>
<tr>
<td>4.25</td>
<td>$15 \pm 2^A$</td>
</tr>
</tbody>
</table>

The superscript letters (A, B) mean that the results within the same column without a common letter are significantly different ($p< 0.05$); the subscript letters (a, b, c, d) mean that the results within the same row without a common letter are significantly different ($p< 0.05$).

**Table 4.3:** Apparent molar binding ratios ($n$, expressed in mol ligand per mol of protein monomer) of nutraceutical model compounds toBLG at pH 7.00 and 4.25.

<table>
<thead>
<tr>
<th>pH</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$-carotene</td>
</tr>
<tr>
<td>7.00</td>
<td>$0.48 \pm 0.04^B_a$</td>
</tr>
<tr>
<td>4.25</td>
<td>$0.23 \pm 0.03^A_a$</td>
</tr>
</tbody>
</table>

The superscript letters (A, B) mean that the results within the same column without a common letter are significantly different ($p< 0.05$); the subscript letters (a, b, c) mean that the results within the same row without a common letter are significantly different ($p< 0.05$).
A low $K'_d$ value indicates high binding affinity. The analysis suggested that binding occurred under all conditions but varied as a function of pH and nutraceutical model. From these observations ($n>1$ as well as binding dependence on pH and nutraceutical type), it can be concluded that the four nutraceutical models investigated are bound on proteins on different binding sites and/or by different binding mechanisms. An opposite trend was observed for the two estimated parameters at both pH values, where the weaker observed affinity was associated with the higher $n$ value.

Among the four nutraceutical models investigated, β-carotene had the highest binding affinity and the lowest binding stoichiometry at both pH values. The more hydrophobic character of β-carotene may explain the higher affinity of this molecule for the hydrophobic binding sites of BLG, whereas its large size may be responsible for the lower binding stoichiometry. Frapin, Dufour and Haertlé (1993) reported that the affinity of BLG for saturated fatty acids increases gradually from lauric acid to palmitic acid (as a longer aliphatic chain) due to an increase in the hydrophobic nature of the ligand. The β-carotene-BLG molar binding ratio at pH 7 was 0.48±0.04 which is in good agreement with the results (0.49±0.03) reported by Dufour and Haertlé (1991). The solvent exposure of a part of the retinol isoprenoid chain, when bound to BLG could explain the 1:2 stoichiometry of β-carotene-BLG complexes. This tetraterpen has two β-ionone cycles, joined by an isoprenoid chain, which may interact distally with two BLG molecules (Dufour & Haertlé, 1991). Another possibility is that this ligand can bind at the dimer interface. According to Wang, Allen and Swaisgood (1998, 1999) the site found at the dimer interface is the highest affinity site as observed in the current study.
Folic acid is less hydrophobic than curcumin and ergocalciferol but is bound to a higher extent. This suggests possible interactions that are not only hydrophobic in nature, and/or binding to the other binding sites than the calyx. Liang and Subirade (2010) proposed that the binding site of resveratrol may be on the outer surface near Trp19-Arg124, while folic acid binds to the surface hydrophobic pocket in a groove between the α-helix and the β-barrel. The n value of folic acid-BLG complex at pH 7 was 1.25± 0.05. This value is in very good agreement with those (1.30 ± 0.03 and 1.17 ± 0.04) obtained by Liang et al. (2010) during excitation at 280 and 295 nm, respectively.

The curcumin-BLG ratio at pH 7 was 0.95± 0.06 which is in good agreement with the results (1 and 0.85) reported by Sneharani, Karakkat, Singh and Rao (2010) and by Mohammadi, Bordbar, Divsalar, Mohammadi and Saboury (2009) at pH 7 and 6.4, respectively. Based on the obtained results (no significant change in binding stoichiometry) at the lower pH level (4.25) and on those reported in literature (Riihimäki, Vainio, Heikura, Valkonen, Virtanen, &Vuorela, 2008), it seems that curcumin (as a phenolic compound) binds to a site other than the calyx.

The ergocalciferol-BLG molar ratio at pH 7 was 1.31± 0.12. Wang, Allen, and Swaisgood (1997) reported the value of 2.00 ± 0.16 at pH 7. It seems that the increased stoichiometry (binding to the external hydrophobic surface patch) is accompanied by relatively loose binding as evidenced by the decreased observed affinity. According to Forrest, Yada, and Rousseau (2005), the weakest affinity site is found at the hydrophobic surface patch.

A higher than 1 stoichiometry indicated that the other binding sites (crevice next to the alpha helix and the dimer interface) of higher or equal affinity are involved in
the binding to BLG. These binding sites may be saturated prior to, or simultaneously with the main binding site. Zimet et al. (2009) reported that 2.67 ± 1.26 moles of DHA were bound per mole of BLG.

The binding capacities of BLG mediated by pH were evident in the present study. Upon lowering the pH, the binding constants changed depending on the nutraceutical nature. Since the EF loop, that acts as a gate in the central cavity, is in a closed conformation at acidic pH (Kontopidis et al., 2004), the binding stoichiometry of β-carotene was decreased with decreasing pH. Previous NMR studies have shown that palmitic acid starts to be released at a pH lower than 6, and 80% of the palmitic acid has already been released at pH 2 (Ragona, Fogolari, Zetta, Perez, Puyol, De Kruiif, Lohr, Ruterjans, & Molinari, 2000). The binding affinity of β-carotene at pH 4.25 was increased because hydrophobic interactions are enhanced at pH values around the isoelectric point of BLG (4.7-5.2). Since the EF loop is blocking ligand access, another possibility is that a single β-carotene molecule was tightly bound between the monomers (at the interfaces) found within the octamers as evidenced by the strongest observed affinity.

The decrease in the binding stoichiometry of folic acid can be attributed to its lower solubility at pH 4.25 than at pH 7 (~1 and ~5 mg/L, respectively) (Younis, Stamatakis, Callery, & Meyer-Stouta 2009).

The binding stoichiometry of curcumin was not significantly (p<0.05) different at pH 4.25 as compared to pH 7.00. Riihimäki et al. (2008) reported that, contrary to retinol, the release of phenolic compounds was not observed at acidic pH, which suggests that phenolic compounds and their derivatives do not bind to the central calyx. Liang et al. (2008) studied the binding of the natural polyphenolic compound
resveratrol to bovine BLG. The observed blue shift of the fluorescence emission maxima and the increase in the emission intensity implied that the environment of the polyphenol bound to BLG is not as hydrophobic as the cavity of BLG, suggesting binding on the surface of the protein. Moreover, the qualitative docking results performed by Riihimäki et al. (2008), showed that phenolic compounds and their derivatives would not bind to the central calyx, supporting the results of this study for curcumin-BLG complex.

The binding stoichiometry of ergocalciferol was significantly decreased by decreasing pH. At pH 4.25, approximately 0.85 molecule of vitamin D$_2$ were bound per BLG monomer with a relatively weak affinity. Since the EF loop is in a closed position at this pH, the results indicate that loose binding occurred at the external hydrophobic surface patch. Since protein association is increased after lowering the pH to 4.25 (formation of the octamers), it seems that the decreased available surface area inhibited greater ligand access at external hydrophobic surface patches and interfaces. The binding to the external hydrophobic surface patches and the interfaces is accompanied by relatively loose and tight affinity, respectively. The absence of a significant change in binding affinity of ergocalciferol to BLG with decreasing pH is likely due to a combination of both a decrease in binding affinity arising from the contribution of the hydrophobic surface patches in binding and an increase in binding affinity caused by the binding to interfaces resulting in an overall unchanged binding affinity.

4.7. NMR results

Nuclear magnetic resonance (NMR) spectroscopy is a useful technique to study molecular interactions. NMR provides different measurable parameters that
depend on the amount and the strength of the interaction. The latter gives rise to important changes in the chemical environment and in the rotational as well as translational mobility of the ligand, which is reflected in a change in the chemical shift, the line width and relaxation rate, as well as the molecular diffusion rate constants of the ligand. Indeed, binding of ligands to hydrophobic sites may cause a change in chemical shift to lower values. In addition, bound ligand molecules will have a reduced mobility which provokes a peak broadening. In addition, the degree of binding may be quantified by analysis of the diffusion behaviour of ligand and protein both separately and upon mixing. Thus, binding of SDS has been clearly shown by 1HNMR. Considering the relatively limited binding of hydrophobic ligands (ranging from 1/2 to 2/1 molar ratio), the large difference in molar mass and the absence of highly intense peaks in the NMR spectrum of the ligands (as compared to the intense CH2-signal in typical surfactant-like chemicals), NMR detection of the ligand in the presence of a large amount of protein is hampered. To overcome this problem, initial measurements were performed using Anilino Naphthalene Sulphonate. This fluorescent dye is widely used as a surface hydrophobicity probe, based on the fact that its fluorescence intensity is largely increased when present in a rather hydrophobic environment. First of all, ANS is known to bind to proteins. In addition, its aromatic nature is responsible for the fact that most ligands NMR peaks are resolved from the protein peaks.1D proton NMR is a quick method to achieve qualitative information about the degree of interaction. In this type of experiment the variation in chemical shift of the ligand resonances upon sorption as compared to the dissolved molecules can be used to roughly estimate the partitioning coefficient as well as the type of interactions. A more accurate quantification of the interaction of the ligands with BLG is obtained by using Diffusion Ordered Spectroscopy (DOSY-
NMR) measurements. Hereby, the observed diffusion coefficient of the ligand in the presence of proteins ($D_{\text{obs}}$) is the weighted average of non-bound molecules (with diffusion coefficient $D_{\text{free}}$) and protein bound molecules (with the same diffusion coefficient as the protein $D_{\text{pro}}$). If the bound fraction is represented by $P$, the weighted average may be calculated according to the following equation:

$$D_{\text{obs}} = PD_{\text{sorb}} + (1 - P)D_{\text{free}} \quad (4-1)$$

from which

$$P = \frac{D_{\text{free}} - D_{\text{obs}}}{D_{\text{free}} - D_{\text{sorb}}} \quad (4-2)$$

Hence, the bound protein fraction follows from experimental values of the diffusion coefficient of the ligands in the absence ($D_{\text{free}}$) and presence ($D_{\text{obs}}$) of protein, as well as from the diffusion coefficient of the protein ($D_{\text{pro}}$).

### 4.7.1. ANS+BLG

![Chemical structure of ANS](image)

**Fig. 4.16:** Chemical structure of ANS

Fig. 4.17 shows the $^1$H-NMR spectrum of 3 mM ANS in D₂O at 25 °C. The spectrum shows 8 signals and a total integrated area of 11 that matches the number of aromatic protons of the molecule.
The triplet at 6.85 ppm is relative to the para proton of the aniline. It should be mentioned that ppm stands for parts per million, and it is the unit used to measure chemical shift. The doublet at 7.06 ppm (integral=2) belongs to the ortho protons of the aniline, while the triplet at 7.23 ppm (integral=2) is due to the meta protons of the aniline. All the other resonances cannot be unambiguously assigned. To get a complete proton assignment, a series of 2D NMR experiments need to be performed such as COSY, TOCSY and NOESY. Fig. 4.18 shows the $^1$H-NMR spectra of 3mM ANS alone, in the presence of 0.25 and 1 mM BLG. In the presence of 0.25 mM BLG the characteristic resonances of ANS are still visible although slightly shifted and much broader. Both these two observations suggest the existence of interactions between the two components. At higher protein concentration (1 mM), the ANS resonances are almost completely covered by the protein ones. The peaks at 4.78 ppm and 1.81 ppm are representative of HDO and sodium acetate (used as internal standard).
**Fig. 4.18:** proton NMR spectra at 25 °C of 3 mM ANS: alone (blue), with 0.25 mM of BLG (red) and with 1 mM BLG (green) in D$_2$O

Fig. 4.19 shows the DOSY spectra of ANS with and without BLG. It is clear from the spectra that the diffusion behavior of ANS is different in the two situations. Based on equation (4-2), it is possible to estimate the fraction of ANS sorbed (P).

**Fig. 4.19:** DOSY spectra of 3 mM ANS in D$_2$O at 25 °C without (blue) and with 0.25 mM BLG (red)
In the absence of protein the diffusion coefficient of ANS is $(4.81 \pm 0.02) \times 10^{-10}$ m$^2$s$^{-1}$. In the presence of 0.25 mM of protein the diffusion of ANS becomes $(3.44 \pm 0.01) \times 10^{-10}$ m$^2$s$^{-1}$. This diffusion coefficient was estimated following the echo decay of the ANS peak at 8.25 ppm because this is less affected by the presence of protein background. The diffusion coefficient of protein is $(0.75 \pm 0.01) \times 10^{-10}$ m$^2$s$^{-1}$. It follows that the fraction of sorbed ANS is 33.7%. Considering the concentrations used for this system (3mM ANS and 0.25mM BLG), this bound fraction corresponds on average to 4.1 ANS molecules per BLG monomer.

4.7.2. Toluene+BLG

![Chemical structure of toluene](image)

*Fig. 4.20: Chemical structure of toluene*

Fig. 4.21 shows the proton NMR spectrum of 3 mM toluene in D$_2$O at 25 °C. Once again the peaks at 4.78 ppm and 1.81 ppm are representative of HDO and sodium acetate (used as internal standard). The peaks at 7.14, 7.19 and 7.24 ppm belong to the aromatic ring protons, para, ortho and meta, respectively. Finally the resonance at 2.25 ppm is due to the methyl group.
Fig. 4.21: Proton NMR spectrum of 3 mM toluene in D$_2$O at 25 °C.

Although a concentration of 3 mM is generally sufficient to give rise to clear NMR resonances, it is evident from the spectrum that the intensities of the toluene resonances are very low. Hence, it is probable that the solubility of toluene in D$_2$O is much lower than in H$_2$O, so that only a small fraction of the toluene is dissolved and hence visible in the NMR spectrum.

Fig. 4.22 shows the NMR spectra of 3 mM toluene alone and with different concentrations of BLG. At the lowest protein concentration (0.25 mM) the signals of the aromatic molecule are slightly visible (red boxes).
Fig. 4.22: NMR spectra of 3 mM toluene in D$_2$O at 25 °C: alone (blue) and with BLG 0.25mM (red) and 1mM (green).

Fig. 4.23 shows the DOSY spectra of 3mM toluene with and without protein, based on mono-exponential fitting of the experimental data. From Fig. 4.23 it seems that toluene diffuses much more slowly in the presence of protein. In this case, because the ligand-protein overlapping, it is not possible to exclusively follow the toluene echo decay. Although the toluene resonances disappeared in the $^1$H-NMR spectrum, because of the overlap with protein peaks, DOSY experiments allow the separation of the two components (i.e toluene and protein) in the second dimension (diffusion dimension) based on their different diffusion behavior.
As consequence, a more reliable estimation of the ligand diffusion coefficient can be obtained by fitting the decay of the signal by means of biexponential fitting as shown in Fig. 4.24. The biexponential decay is characterized by two diffusion behaviors: the fast is related to the toluene while the slow one is due to the protein contribution. From this fitting, the toluene diffusion coefficient in the presence of protein (D_{obs}) was estimated to be $(5.82 \pm 0.40) \times 10^{-10} \text{m}^2\text{s}^{-1}$. The free diffusion coefficient (D_{free}) of toluene and the diffusion coefficient of protein are $(8.04 \pm 0.08) \times 10^{-10} \text{m}^2\text{s}^{-1}$ and $(0.75 \pm 0.01) \times 10^{-10} \text{m}^2\text{s}^{-1}$, respectively. From these values the solubilized fraction was calculated to be 30.5%. This bound fraction corresponds on average to 3.7 toluene molecules per BLG monomer.
Fig. 4.24: Biexponential fitting of the echo decay of the signal for the sample containing 3 mM toluene as well as 0.25 mM whey protein.

### 4.7.3. Catechin + BLG

![Catechin chemical structure](image)

Fig. 4.25: (+) Catechin chemical structure

Fig. 4.26 shows the $^1$H-NMR spectrum of catechin. Also in this case, only few signals can be assigned, in particular the diastereotopic methylene protons and the methyn protons of the pyran ring at 2.5 ppm and 4.14 ppm, respectively. All the others require a more in depth characterization to be unequivocally assigned.
In Fig. 4.27 the spectra of catechin with and without protein are presented. Upon protein addition the catechin resonances are all still visible, but slightly shifted and somewhat broader, which is reflected in the fact that less fine structure (due to subdivision of some NMR contributions in doublets, triplets, etc.) can be seen in the presence of the whey proteins. These two effects suggest the presence of interaction between the catechin and the BLG.
Fig. 4.27: NMR spectra of 4 mM catechin in D$_2$O at 25 °C: alone (blue) and with BLG 0.25 mM (red)

The diffusion spectra, on the other hand, show no significant variation of diffusion behavior of the catechin in presence and absence of protein (Fig. 4.28). Hereby, it is important to realize that even if binding would occur with a 1/1 molar ratio, only 1/16 of all the epicatechin could become protein bound at most, so that it becomes less obvious to see a clear change in diffusional behaviour. Whereas increasing the protein concentration might help to increase the bound fraction and hence decrease the observed diffusion coefficient, it would at the same time complicate reliable measurements by exaggerating the background signal of the protein. Hereby, it also has to be realized that the 16/1 molar ratio of catechin to protein corresponds to a 0.252 weight ratio, which explains the significant protein contribution in the NMR spectrum of mixed samples.
Fig. 4.28: DOSY spectra of 4 mM catechin in \( \text{D}_2\text{O} \) at 25 °C: alone (blue) and with BLG 0.25 mM (red)

4.7.4. Folic acid+BLG

![Folic acid chemical structure](image)

Fig. 4.29: Folic acid chemical structure

In this case a complete assignment is possible as summarized in Fig. 4.30. From Fig. 4.31, it is clear that the addition of protein only slightly influences the NMR contribution of the folic acid. The resonances show little chemical shift variation and there are no signs of line broadening. However, the diffusion measurements show
that the diffusion behavior of folic acid changes (slower diffusion) upon addition of protein, as visible in Fig. 4.32. The sorbed amount of folic acid can be estimated according to the previously mentioned equation, where $D_{\text{free}}$ is $(3.19 \pm 0.04) \times 10^{-10}$ m$^2$s$^{-1}$, $D_{\text{obs}}$ is $(2.96 \pm 0.05) \times 10^{-10}$ m$^2$s$^{-1}$ and $D_{\text{pro}}$ is $(0.75 \pm 0.01) \times 10^{-10}$ m$^2$s$^{-1}$. The solubilized fraction is 9.4%, which corresponds on average to 1.1 folic acid molecules per BLG monomer. This result is in agreement with the results of fluorimetry.

**Fig. 4.30:** NMR spectrum of 3 mM folic acid in D$_2$O at 25 °C with relative peak assignment
The same series of experiments were repeated for the same system but at different pH conditions (pH ≈ 4.25). Unfortunately, in these conditions, the NMR spectrum resulted in a complete loss of the folic acid resonances. As a further
consequence, the diffusion experiments were not possible. Wu, Li, Hou, & Qian (2010) reported the solubility values of folic acid at different pH conditions. The authors found that around pH 4, the solubility is very low, i.e. 1.02 mg/L. This very poor aqueous solubility is the main reason of the loss of folic acid resonance at lower pH conditions.

In this section we investigated the usefulness of NMR techniques to investigate ligand-BLG systems. 1D proton experiments can be used as quick screening method to probe the possible interactions between ligand and BLG. Moreover, a more accurate analysis of the chemical shift variations also allows revealing which ligand functional group is more in contact with protein and as consequence to establish the nature of the interactions. In addition, diffusion NMR was used to evaluate quantitatively the fraction of ligand directly bound to the protein. However, this technique can only be used provided that the ligand has a sufficiently large solubility (i.e. at least some mM) in (heavy) water. Hence, NMR could not be used to study the binding of β-carotene, curcumin or ergocalciferol.

4.8. Nanoencapsulation and colloidal stability of nutraceutical models

The effects of BLG and polysaccharide (ALG) on the colloidal stability of nanoencapsulated curcumin (as hydrophobic model) and folic acid (as hydrophilic model with low solubility at acidic pH) at pH 4.25 are shown in Fig. 4.33 and 4.34, respectively.
Fig. 4.33: Effects of curcumin nanoencapsulation on its colloidal stability at pH 4.25 (I: dissolved curcumin in ethanol added to deionized water; II: dissolved curcumin in ethanol added to BLG dispersion (0.1% w/w); III: dissolved curcumin in ethanol added to BLG-ALG soluble complexes (ALG/BLG weight ratio of 0.75).

Except for the blank sample of curcumin (which was first dissolved in ethanol before being added to the aqueous phase) in the deionized water, there was not any significant difference between the samples just after production (Fig. 4.33.A and 4.34.A). After 2 hours of production, some differences were observed. The curcumin and folic acid, incorporated into deionized water, started to precipitate and separated almost completely after 24 hours of production.
Fig. 4.34: Effects of folic acid nanoencapsulation on its colloidal stability at pH 4.25 (I: dissolved folic acid in deionized water added to deionized water; II: dissolved folic acid in deionized water added to BLG dispersion (0.1% w/w); III: dissolved folic acid in deionized water added to BLG-ALG soluble complexes (ALG/BLG weight ratio of 0.75).

The samples containing BLG and BLG+ALG did not show any precipitation after 24 hours (Fig. 4.33.B and 4.34.B). However, the turbidity of both samples was slightly different. 48 hours after production, the differences between these two samples were more obvious (Fig. 4.33.C and 4.34.C): the blank sample containing the nutraceutical models and BLG showed some precipitation, while the main sample, which contained the nutraceutical models, BLG and ALG, remained completely transparent without
any sign of precipitation. This clearly demonstrated the efficacy of soluble complexes arising from protein-polysaccharide interactions on nanoencapsulation and colloidal stability of nutraceuticals of low solubility in water even 30 days after production without storing in the dark (Fig. 4.33.D and 4.34.D). The low solubility of curcumin in aqueous solution significantly limits its application. Recently, it has been shown that polyelectrolyte-coated curcumin nanoparticles (obtained through a layer by layer shell assembly) are hydrosoluble (Zheng, Zhang, Carbo, Clark, Nathan, & Lvov, 2010). In our study, curcumin binding to BLG increased its colloidal stability in water. The stability of the main samples containing folic acid was lower than those containing curcumin. The nutraceutical models were incorporated in a 1:1 molar ratio into the protein solution. The apparent mole ratio of folic acid to BLG at pH 4.25 was determined to be 0.39 ± 0.04 (Table 4.3). Hence, it is possible that after decreasing the pH to 4.25, the BLG became overloaded and some precipitation occurred. As the apparent mole ratio of curcumin to BLG at pH 4.25 was found to be 0.82 ± 0.08 (Table 4.3), BLG overloading was relatively prevented in this case. The particle size and electrophoretic mobility analyses of the samples containing BLG (0.1% w/w) and ALG (0.075 % w/w) with and without nutraceutical model compounds showed that curcumin addition into the soluble complexes of BLG-ALG, slightly increased the particle size from 269 nm to 278 nm and also slightly decreased the electrophoretic mobility from -4.52 to -4.30 (10^{-8} m^2/Vs). Folic acid incorporation into BLG-ALG soluble complexes, increased the particle size from 269 nm to values higher than 1µm (maybe due to the low solubility of folic acid at pH 4.25) and also decreased the electrophoretic mobility from -4.52 to -3.95 (10^{-8} m^2/Vs). These experimental values are in good agreement with the qualitative visual observations, which indicated an increased turbidity in the presence of folic acid. A similar decrease in the absolute
value of the electrophoretic mobility was reported by Zimet et al. (2009) upon docosahexaenoic acid addition into BLG-pectin soluble complexes. These results showed that the delivery systems formed can be used for fortification purposes of transparent acidic beverages with both hydrophobic and hydrophilic bioactives. In this study, the intrinsic transporting properties of BLG were utilized to develop nano-sized green delivery systems.

Regarding bioprotection efficiency, it has been reported that BLG can form water-soluble complexes with DHA and ergocalciferol and protect these lipophilic compounds from degradation by heat and oxidation (Zimet et al., 2009; Ron et al., 2010). Liang et al. (2008) and Forrest et al. (2005) concluded that interaction with BLG may strongly influence the stability of phenolic compounds and vitamin D₃ and hence their bioavailability in processed foods. It has been shown that BLG has some antioxidant activity, apparently due to its free thiol group (Liu, Chen, & Mao, 2007). Therefore, BLG can be used as a versatile carrier of bioactive molecules in controlled delivery applications.

Another important attribute of a suitable delivery system is its stability against processing conditions (such as thermal processing) which can result in protein denaturation and hence affecting its transporting properties. The precise denaturation process is complex and is influenced by factors such as pH, protein concentration, ionic environment, genetic variant and presence of ligands. Both lowering the pH (Relkin, Eynard, & Launay, 1992) and adding calyx-bound ligands (Busti, Gatti, & Delorenzi, 2006) make the protein more resistant to thermal unfolding. Enzymatic proteolysis observations indicate that BLG is less susceptible to pressure induced changes at acidic pH than at neutral or basic pH (Edwards et al., 2009).
The targeted delivery and controlled release are also very important. As an example, it is beneficial for the encapsulated folic acid to be released in the small intestine where most of the absorption of vitamins takes place. The jejunum is the site of maximum absorption of free folates, where absorption occurs by a pH dependent, carrier-mediated system (Kailasapathy, 2008). Therefore, the biopolymers used for encapsulation should be able to protect the folate in the upper gastrointestinal tract (acidic stomach conditions) and release the folate in the alkaline conditions in the small intestine. The BLG structure is relatively compact and stable in aqueous solutions at acidic pH, as demonstrated by its resistivity to proteolysis by pepsin (Mohan Reddy, Kella, & Kinsella, 1988). The compact structure of BLG at acidic pH can be overprotected by using an anionic polysaccharide due to the electrostatic interactions. The BLG-anionic polysaccharide complexes will be dissociated at alkaline pH of the small intestine.
5. Conclusion

Proteins and polysaccharides are two main structural entities in foods and their complexes and coacervates are powerful and versatile tools to produce tailor-made food microstructure of promising structural features. During last decade, there have been many advances made in the field of protein–polysaccharide complexes and coacervates enabling to identify the most important physicochemical parameters (pH, ionic strength, biopolymer mixing ratio, total biopolymer concentration, and polymer charge density) controlling complex formation. The present work showed that ultrasound irradiation can effectively depolymerize sodium alginate and κ-carrageenan. The rate of depolymerization was dependent on the amplitude, time and temperature of sonication. Polysaccharide sonication decreased its affinity constant and/or binding strength to β-lactoglobulin at pH 4.25 as determined by ITC and SCD. The properties of the biopolymer nanoparticles formed depended strongly on the polysaccharide type and concentration as shown by DLS and ζ-potential analyses. The soluble nanocomplexes formed had a good stability against aggregation.

Many beneficial food bioactives such as carotenoids, fat-soluble vitamins and phenolic compounds are lipophilic. The hydrophobic nature of these compounds makes their incorporation into aqueous foods and beverages challenging. The use of protein–polysaccharide complexes and coacervates as delivery systems for nutraceuticals in liquid foods is very attractive owing to the variety of biopolymer couples which could be adapted to the various delivery requirements (e.g. stability, mechanical properties and controlled release). The biopolymers which form complexes or coacervates can themselves constitute the bioactive agent (such as bioactive peptides). In this study, the intrinsic transporting properties of BLG were
utilized to develop nano-sized green delivery systems. The binding analysis suggested that the binding occurred under all conditions but varied as a function of pH and nutraceutical model compound. From those observations, it could be concluded that the four nutraceutical models investigated are bound to proteins on different binding sites and/or by different binding mechanisms. The binding capacities of BLG mediated by pH were evident in the present study. Upon lowering the pH, the binding constants changed depending on the nutraceutical nature. Whereas NMR enables a more direct determination of the binding to proteins, this technique suffers from the fact that it can only be applied for ligands with a sufficiently large solubility (i.e. in the mM range) in the aqueous phase. These findings resulted in designing nanoscopic delivery systems for encapsulation of both hydrophilic and hydrophobic bioactives in liquid food products. The preliminary stability experiments demonstrated the efficacy of soluble complexes arising from protein-polysaccharide interactions on nanoencapsulation and colloidal stability of nutraceuticals of low solubility in water. These results showed that the delivery systems formed can be used for fortification purposes of transparent acidic beverages (such as apple juice and herbal beverages) with both hydrophobic and hydrophilic bioactives.

The main drawback of protein-polysaccharide complexes and coacervates is the sensitivity of their functional properties to pH. However, considering the large spectrum of protein-polysaccharide couples available there are numerous possibilities to obtain the desired effect at a determined ratio (Turgeon et al., 2009). Exploring protein interaction with a mixture of polysaccharides of low and high molecular weights and vice versa would be interesting. Protein modification and hydrolysis prior to complexation are the other important areas of research. At
present, our understanding of the structure-function relationships is limited. In the future, more detailed information is required about the morphology of the stable soluble nanocomplexes formed using high-resolution cryo-transmission electron microscopy (cryo-TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM). Another important research field is the optimization of multi-ingredient systems. Designing ‘intelligent’ nanoscale encapsulation systems for food and pharmaceutical applications using multilayered nanoparticles is another interesting area of research. Last but not least, additional researches on the heat stability of the nanocomplexes, the bioprotection conferred to the encapsulated bioactives during heat treatment (industrial pasteurization and cold storage), the sensory properties of the final product and the bioavailability of the bioactive compounds are surely needed to convince the food industry of the usefulness of this encapsulation technology. Taking into account the cost and availability of food ingredients, the only way to take the full potential advantage of these interesting colloids is fitting the complex formation conditions obtained for “pure systems” with “real systems” and also adapting to the food processing conditions.
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Curriculum Vitae

Personal Data

Name Seyed Mohammad Hashem Hosseini
Address No. 48, East Ghoddousi St., Shiraz, Iran
Telephone +98 917 736 2953
E-mail hhosseini@ut.ac.ir; hhosseini10@gmail.com
Date of birth 19/09/1982
Place of birth Jahrom
Nationality Iranian
Marital status Married to Elaheh Bahari
1 child: Danyal (2011)
Education

♦ Joint PhD: Food Science and Applied Biological Sciences (Complex coacervation between β-lactoglobulin and anionic polysaccharides and its potential application for nanoencapsulation of hydrophobic functional components), Food Science and Technology Department, University of Tehran, Karaj, Iran and Particle and Interfacial Technology Group (Paint), Faculty of Biosciences Engineering, Ghent University, Gent, Belgium (September 2013)

  o Title of qualification awarded: Best Grade Student in the Entrance Examination and Courses

♦ M.Sc.: Food Science and Technology (Antimicrobial, physical and mechanical properties of chitosan-based edible films incorporated with thyme, clove and cinnamon essential oils), Food Science and Technology Department, University of Tehran, Karaj, Iran (September 2007).

♦ B.Sc.: Food Science and Technology, Food Science and Technology Department, University of Shiraz, Shiraz, Iran (July 2005).
Research Interests

Techno-functional properties of protein-polysaccharide interactions, Biopolymer based antimicrobial edible films and coatings

Publications


Selected papers published in peer-reviewed journals


Other Publications


