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REVIEW



## Interactions of salivary mucins and saliva with food proteins: a review

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### ABSTRACT

Mucins are long glycoprotein molecules responsible for the gel nature of the mucous layer that covers epithelial surfaces throughout the body. Mucins, as the major salivary proteins, are also important proteins for the food oral processing and digestion. The interactions of salivary mucins and saliva with several food proteins and food protein emulsions, as well as their functional properties related to the food oral processing were reviewed in this paper. The target food proteins of focus were whey proteins (lactoferrin and beta-lactoglobulin) and non-whey proteins (casein, gelatin, galectin/lectin, and proline-rich proteins). Most of the studies suggest that electrostatic attraction (between positively charged food proteins with negatively charged moieties of mucin mainly on glycosylated region of mucin) is the major mode of interaction between them. On the other hand, casein attracts the salivary proteins only via non-covalent interactions due to its naturally self-assembled micellar structure. Moreover, recent studies related to  $\beta$ -lactoglobulin (BLG)-mucin interactions have clarified the importance of hydrophobic as well as hydrophilic interactions, such as hydrogen bonding. Furthermore, *in vitro* studies between protein emulsions and saliva observed a strong aggregating effect of saliva on caseinate and whey proteins as well as on surfactant-stabilized emulsions. Besides, the sign and the density of the charge on the surface of the protein emulsion droplets contribute significantly to the behavior of the emulsion when mixed with saliva. Other studies also suggested that the interactions between saliva and whey proteins depends on the pH in addition to the flow rate of the saliva. Overall, the role of interactions of food proteins and food protein emulsions with mucin/saliva-proteins in the oral perception, as well as the physicochemical and structural changes of proteins were discussed.

### KEYWORDS

$\beta$ -lactoglobulin; whey; casein; gelatin; lactoferrin; lectins; emulsion

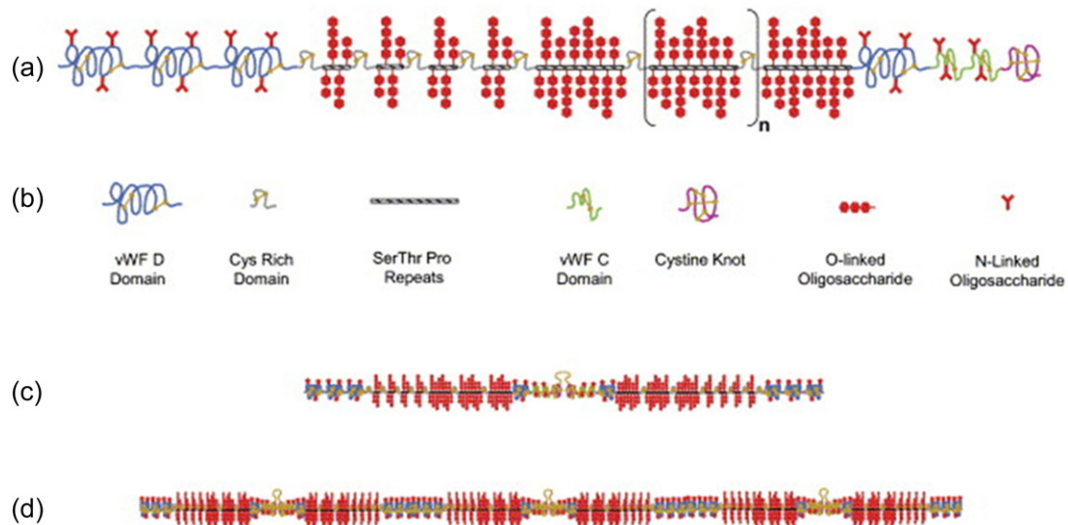
### Introduction

A mucous layer covers epithelial surfaces throughout the body and provides protection and lubrication of underlying epithelium.

The main non-water components of the mucous layer are mucins, long glycoprotein molecules that are responsible for the gel nature of the mucous layer (Efremova et al. 2002). Mucin is a glycoprotein consisting of a linear polypeptide core with a highly glycosylated central part accounting for up to 80% of its molecular weight which ranges between 0.5 and 20 MDa (Shi et al. 2000; Bansil and Turner 2006). Due to the abundance of negatively charged groups, arising mainly from sialic acid residues and sulfated sugars, mucins generally have low isoelectric points, estimated to be between 2 and 3. The presence of a large number of charged groups results in pH-dependent physicochemical properties of mucins. Mucins are amphiphilic due to alternating arrays of negatively charged, hydrophilic glycosylated regions (enriched with serine, threonine, and proline residues) and hydrophobic unglycosylated patches (enriched with cysteine residues) (Figure 1). Monomeric mucin molecules can be linked with each other, such as via disulfide bonds by

cysteine residues to form larger aggregates (Durrer et al. 1995; Lee et al. 2005). Mucin's cysteine residues are capable of forming intermolecular disulfide bonds with other proteins as well (Mehrotra, Thornton, and Sheehan 1998; Zalewska et al. 2000). Additionally, mucins can also form aggregates with other proteins via non-covalent interactions by involving unglycosylated regions or oligosaccharide side chains.

Furthermore, a first target for oral drug delivery is the mucus layer since it is covering the main target cells. Mucoadhesive drug delivery vehicles and methods to determine mucoadhesive properties have received considerable attention in the last two decades (Lai, Wang, and Hanes 2009; Di Silvio et al. 2015). Mucoadhesive delivery vehicles are designed to exploit the attraction between the mucous layer and the polymer carrier of the drug delivery system. The main advantages of mucoadhesive polymer carriers include the localization of the carriers to a specific site within the body and the prolonged time of delivery. These features greatly enhance the bioavailability of drugs, especially for peptide and protein delivery (Peppas and Robinson 1995; Lehr 1994). Moreover, apart from mucin's functions in biological systems, other studies have shown



**Figure 1.** (a) A schematic drawing of the pig gastric mucin (PGM) monomer consisting of glycosylated regions flanked by regions with relatively little glycosylation. (b) The symbols indicate the different domains in the sketch in (a). (This representation is based in part on Figures 1 and 2 of Dekker et al. (2002)). At the bottom of the figure showing (c) a dimer formed by two monomeric subunits linked via disulfide bonds and in (d) dimers that are further disulfide linked to form higher multimers. This gives rise to the high molecular weight and polydispersity of secretory mucins. (Bansil and Turner, 2006).

facile adsorption and effective lubrication on various engineering materials (Lee et al. 2005; Nikogeorgos, Madsen, and Lee 2014; Yakubov et al. 2009).

Mucin, as the major salivary protein, is also an important protein related to the food oral processing and digestion. Through the various stages of oral processing, foods continuously mix with saliva, mucins interact with food components and the humans perceive various sensory and textural attributes. Food oral processing is a complex mechanical process including chewing, mastication, transportation and swallowing, and involves various soft and hard tissues such as palate, teeth and tongue in mouth. Therefore, it should be investigated in a step-by-step manner, starting with basic components which contribute to the oral processing. Food oral processing can be divided into three stages. The first stage of oral processing, relating to bulk properties such as hardness, brittleness, springiness of food compounds, etc., depends on their rheological properties. The last stage, relating to surface and lubrication behavior such as smoothness, slipperiness, creaminess, etc, depends on the tribological properties. Between these two stages, there is a transition stage, which is dependent on both bulk rheology and surface tribology factors, such as food thickness, consistency and creaminess. It is believed that the fundamental cause of changing sensory intensity and sensory profile is due to the rheology-tribology transition (Chen and Stokes 2012). Tribology measurements have been developed to measure friction by mimicking the rubbing contact in oral cavity lubricated with mucin/saliva and/or food protein solution/emulsion. Sotres and Arnebrant (2013) defined the friction as the resistance offered to the sliding of one surface over another and expresses itself as a (friction) force opposing the sliding. Different factors such as adhesion, interlocking of asperities and surface deformation can also cause this resistance (Sotres and Arnebrant 2013). To analyze the interaction between food and salivary components,

environmental conditions, mainly temperature and pH, are also important to consider.

Despite a number of studies addressing the interactions of mucins with polysaccharides (Menchicchi et al. 2014, 2015; Qaqish and Amiji 1999), studies related to the interaction of mucins with food proteins are still limited (Senapati, Das, and Batra 2010). Hence, in the present paper, the interactions of mucin/saliva with several food proteins and food protein emulsions as well as their functional properties related to the food oral processing are reviewed.

### Mucin/saliva interaction with whey proteins

Whey proteins include  $\beta$ -lactoglobulin (BLG), bovine serum albumin,  $\alpha$ -lactalbumin, lactoferrin, and immunoglobulins. BLG represents about 50% of the total whey proteins in bovine milk. In contrast to caseins, whey proteins possess high levels of secondary, tertiary and, in most cases, quaternary structures (Edwards, Creamer, and Jameson 2009; Kinsella and Whitehead 1989). The important functional properties of whey protein products include water binding, emulsification, foaming and whipping, gelation and nutritional properties (Singh 2011). Emulsion-type products, e.g. coffee whiteners, whipped toppings, cream liqueurs, dietary formulations, liquid nutritional products and medical foods are an important application of caseinates and whey proteins in the food industry.

By means of trained panelists and viscosity measurements, Beecher et al. (2008) showed that electrostatic interaction between positively charged whey proteins and negatively charged saliva proteins caused astringency. Similarly, Jöbstl et al. (2004) proposed that the perception of astringency is closely related with the complexation and precipitation of the astringent compounds with salivary proline-rich proteins, which increase friction in the mouth.

Sano et al. (2005) also showed that whey protein isolate was more astringent at pH 3.5 than gelatin based on trained panelists and taste sensors. They attributed this difference to that whey proteins are more positively charged at pH 3.5 than gelatin. Moreover, they suggested that astringency was due to that whey proteins (pH 3.5) mixed with saliva (pH 7.0) form a slightly acidic solution (pH 5.0) in which whey proteins precipitate. They have explained the formation of aggregates, as indicated by an increased turbidity of whey protein-saliva mixtures, based on two possible mechanisms. First, as proposed by Sano et al. (2005), mixing of whey proteins at acidic pH with saliva at neutral pH could result in a beverage-saliva solution at a pH close to the isoelectric point (pI) of whey proteins, favoring aggregation as observed by an increase in turbidity. In this case, whey protein aggregates are the main source of astringency. Alternatively, it could be due to the electrostatic interactions between the positively charged whey proteins and negatively charged saliva proteins. If salivary proteins have the pIs lower than the pIs of whey proteins, there will be a pH range that results in a net electrostatic attraction and aggregation. Guo et al. (2006) sequenced 5,338 distinct peptides, representing 1,381 distinct proteins, from human saliva. Approximately 3,400 of the 5,338 peptides were identified as having mean pIs lower than 5.0. These proteins are likely to be involved in the electrostatic attractions with whey proteins in acidic beverages. For example, at pH 6.8, both the salivary and whey proteins are highly negative and they are less likely to aggregate. In contrast, at pH 3.4, the net attraction between salivary and whey proteins appears to be the strongest, resulting in the highest level of astringency.

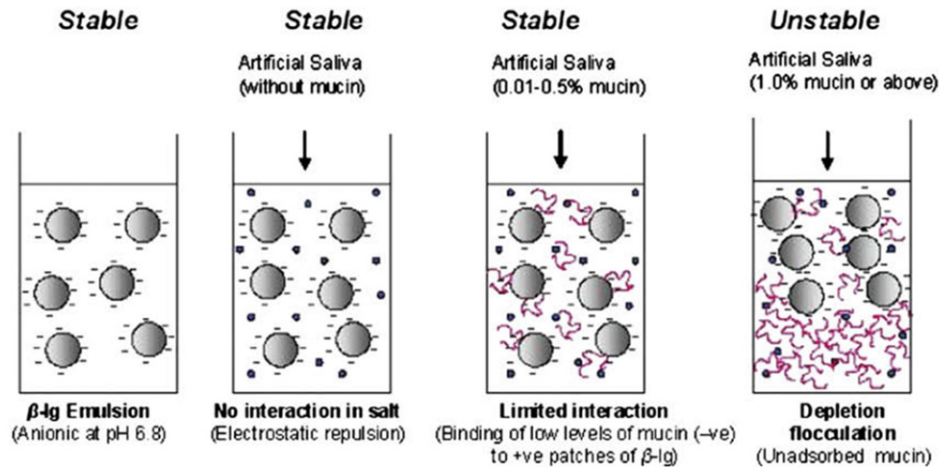
### **Mucin interaction with lactoferrin**

Globular proteins derived from milk are widely used as natural emulsifiers to enhance the formation and stability of oil-in-water emulsions, e.g., whey protein, BLG,  $\alpha$ -lactalbumin, and bovine serum albumin (Dickinson 2003; Kralova and Sjoblom 2009; Livney 2010; McClements 2004; Raikos 2010). Recently, there has been increasing interest in utilization of lactoferrin as an emulsifier (Sarkar et al. 2009; Sarkar, Goh, and Singh 2010). Lactoferrin is a globular glycoprotein derived from milk and other mammalian fluids that has an unusually high isoelectric point (pI >8), and thus cationic at neutral pH whereas most other major dairy globular proteins are anionic (Steijns and van Hooijdonk 2000). The ability of lactoferrin to form positively charged droplets at neutral pH could have a number of important practical implications. Since cationic droplets do not attract positively charged transition metal ions that catalyze oxidation to the droplet surfaces, they are more stable to lipid oxidation than anionic droplets (Mei, McClements, and Decker 1999; Mei et al. 1998). On the other hand, cationic droplets may interact with other anionic ingredients in foods, resulting in the formation of undesirable precipitates (Guzey and McClements 2006). They may also interact with

anionic mucin molecules in the mouth causing astringency (Beecher et al. 2008; Vardhanabhuti et al. 2010).

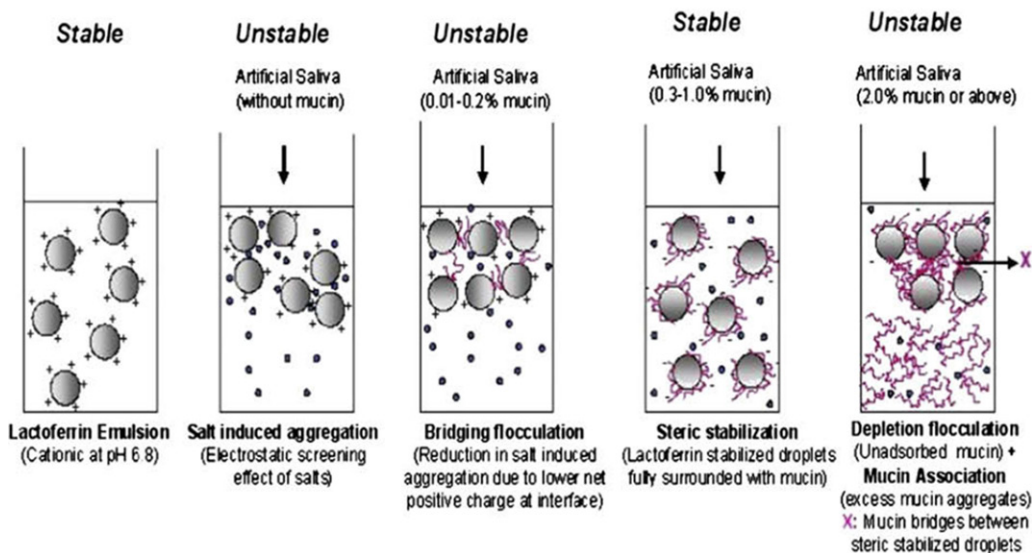
In order to compare cationic and anionic emulsions, BLG-stabilized emulsions and lactoferrin-stabilized emulsions with mucin/saliva have been investigated. When these emulsions were mixed with artificial saliva, containing a range of mucin concentrations and salts, negatively charged mucin was shown to interact more readily with the positively charged lactoferrin-stabilized emulsion droplets to provide a mucin coverage of approximately 1 mg/m<sup>2</sup> (Sarkar et al. 2009). The higher mucin coverage in the lactoferrin-stabilized emulsion was attributed to the electrostatic attraction between oppositely charged mucin molecules (negative) and lactoferrin molecules (positive) adsorbed at the droplet surface. At low levels of mucin addition (0.1–0.2 wt%) to the lactoferrin-stabilized emulsions, the size of the droplet aggregates appeared to decrease and the emulsions appeared to be rather monodisperse at intermediate mucin concentrations (0.5 wt%). However, at higher mucin concentrations (2.0 wt%), the lactoferrin-stabilized emulsions showed aggregation of the oil droplets. To monitor the difference between anionic and cationic emulsions in the physiological condition, the mechanisms of interaction for both BLG-stabilized and lactoferrin-stabilized emulsions in the presence of artificial saliva containing different levels of mucin were illustrated by Sarkar et al. (2009) (Figure 2). Sarkar et al. (2009) suggested that lactoferrin-stabilized emulsion droplets interacted with mucin via electrostatic interactions. They showed that some bridging type flocculation occurred when there was insufficient mucin to form a complete secondary layer around the lactoferrin-stabilized droplets. On the other hand, depletion type flocculation as well as more complex aggregations involving the self-association of mucin molecules increased due to excessive mucin concentration in the continuous phase. These kinds of emulsion-saliva electrostatic interactions might occur upon consumption of emulsions in real situations and could result in different sensorial and textural perceptions *in vivo* (Sarkar et al. 2009; Singh and Sarkar 2011; Singh and Ye 2013).

In view of emulsion stability, Tokle, Lesmes, and McClements (2010) studied the effects of three anionic polysaccharides (namely low methoxyl pectin (LMP), high methoxyl pectin (HMP) and alginate) on the physicochemical properties and stability of lactoferrin-coated lipid droplets. LMP, HMP and alginate were shown to adsorb to the surfaces of lactoferrin-coated droplets at neutral pH, which was primarily attributed to the electrostatic attraction between anionic groups on the polysaccharide molecules and cationic patches on the protein surfaces. The study by Tokle, Lesmes, and McClements (2010) further supports the electrostatic interaction between negatively charged glycosylated regions of mucin with positively charged lactoferrin. Similarly, Vardhanabhuti et al. (2010) showed that lactoferrin was astringent at pH 7.0 where no acid was added, when comparing among various whey proteins isolates (WPI) and lactoferrin at pH 3.5, 4.5, and 7.0. In contrast, astringency of all WPI decreased at pH 7.0. Vardhanabhuti et al. (2010) explained this as a result of that lactoferrin remained positively charged

(a)  $\beta$ -lg-stabilized emulsion-artificial saliva mixtures

## (b)

## Lactoferrin-stabilized emulsion-artificial saliva mixtures



**Figure 2.** Mechanisms of interaction in emulsion-saliva mixtures. Big shaded circle represents emulsion droplets; small solid dot represents salivary salts and coil structure represents mucin molecules. (a) BLG-stabilized emulsion interacting with artificial saliva (b) lactoferrin-stabilized emulsion interacting with artificial saliva. (Sarkar et al. 2009).

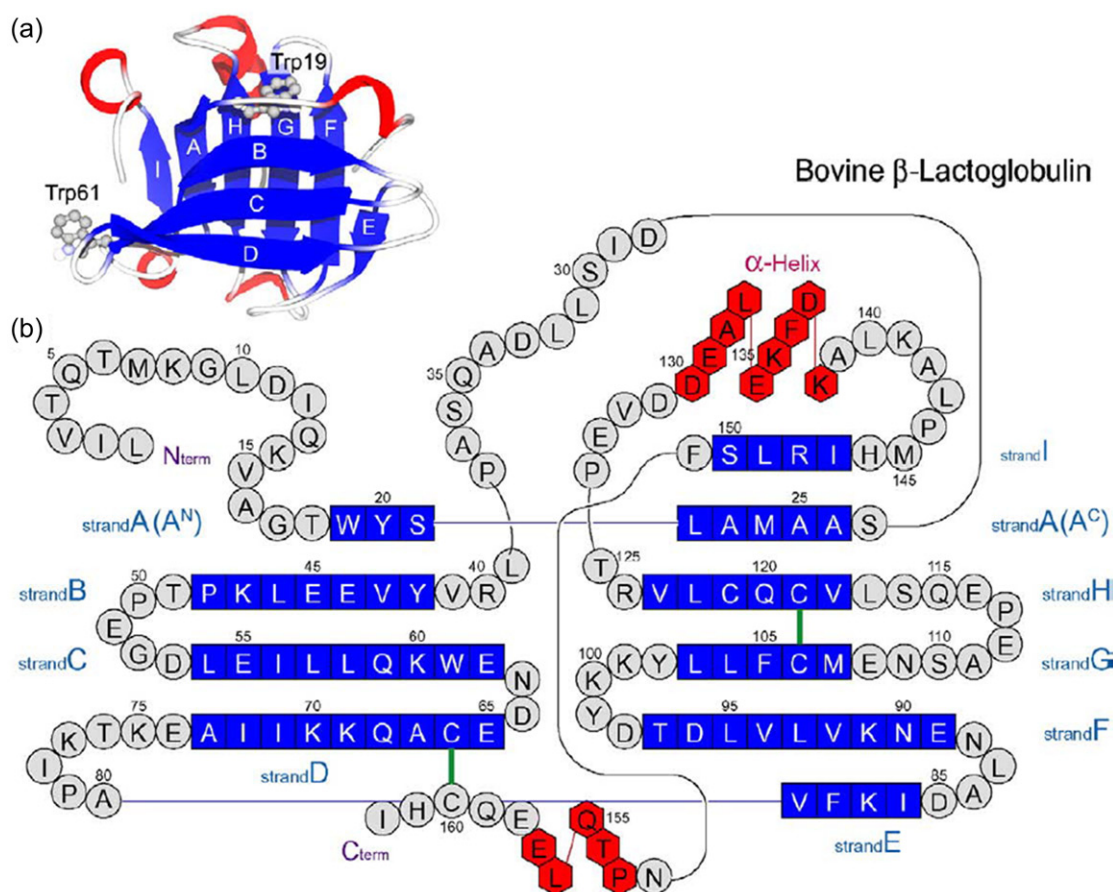
at pH 7.0. It should be noted that while lactoferrin was able to interact with negatively charged saliva proteins, the negatively charged WPI would not interact. Charge interactions were further supported by BLG or lactoferrin and salivary proteins precipitated when mixed at conditions where BLG, lactoferrin, or saliva themselves did not precipitate.

Moreover, Zhang et al. (2015) hypothesized that the presence of mucin in the simulated saliva fluids altered the charge characteristics of the protein-stabilized emulsion systems. Presumably, anionic mucin molecules adsorbed to the surfaces of cationic protein-coated lipid droplets, making the net charge negative. To support this hypothesis, they have measured the electric charge of emulsions after exposure to simulated gastric fluids with and without mucin. In the absence of mucin, the zeta-potentials of emulsions stabilized by caseinate and lactoferrin were 4.5 and 5.7 mV,

respectively. These results suggest that the negative charge of the protein-coated droplets observed in the stomach phase is due to adsorption of mucin molecules to their surfaces.

#### Mucin/saliva interaction with $\beta$ -lactoglobulin (BLG)

$\beta$ -lactoglobulin (BLG) is one of the most important and extensively studied proteins of dairy food systems. BLG is the major whey protein constituting >50% of the total whey proteins in bovine milk. BLG is a typical globular protein with molecular weight of 18.3 kDa and a radius of approximately 2 nm (Zúñiga et al. 2010). According to its amino acid sequence and three-dimensional structure, BLG belongs to the lipocalin family, which can bind to small hydrophobic ligands and may thus act as specific transporters



**Figure 3.** The 3D structure and amino acid sequence of bovine BLG. (A) Ribbon diagram of a single subunit of bovine BLG lattice X, whose pdb code is 1BEB. The  $\beta$ -strands are labeled. Trp residues are represented as balls and sticks. The diagram was produced using the program MolFeat (FiatLux, Tokyo, Japan). (B) A schematic representation of the amino acid residues of the BLG sequence. Residues making up the  $\alpha$ -helix,  $\beta$ -sheet, and loop are represented by hexagons in red, squares in blue, and circles in gray, respectively. Green lines indicate the positions of disulfide bonds. It is seen that BLG has two  $\beta$ -sheets; the B–D strands and N-terminal half of the A strand (denoted AN) consist of one and the E–H strands and C-terminal half of the A strand (denoted AC) consist of the other. (Sakurai et al. 2009).

(Kontopidis, Holt, and Sawyer 2004). It is a predominantly  $\beta$ -sheet protein. The  $\beta$ -barrel, or so-called calyx, is conical and is made of two  $\beta$ -sheets: the B–D strands and N-terminal half of the A strand (denoted AN) form one sheet, and the E–H strands and C-terminal half of the A strand (denoted AC) form the other (Figure 3). On the outer surface of the  $\beta$ -barrel, between the G and H strands, is the 3-turn  $\alpha$ -helix. The loops that connect the  $\beta$ -strands at the closed end of the calyx, BC, DE, and FG, are generally quite short, whereas those at the open end, AB, CD, EF, and GH, are significantly longer and more flexible (Brownlow et al. 1997). In the calyx, there is a large central cavity which is surrounded by hydrophobic residues and is accessible to solvent. This cavity provides the principal ligand-binding site. BLG contains two tryptophan residues, Trp 19 on the A strand and Trp 61 on the C strand. The former is buried in the hydrophobic core whereas the latter is exposed to the solvent in the native structure, making them useful probes for monitoring site-specific conformational changes. BLG contains many charged groups and its structure and properties therefore depend strongly on the pH and ionic strength (Fang and Dalgleish 1997). Taulier and Chalikian (2001) addressed six pH-dependent structural states of BLG depending on solution conditions, and characterized the acid- and base-induced

conformational transitions between these structural states over the pH range from 1 to 13. This pH-dependent system may indicate that one of BLG functions is to bind to non-polar molecules and transport them through the acidic environment (stomach) into the basic environment (intestine). This strong pH dependency suggests that electrostatic interactions could play a significant role in the interactions of BLG with other molecules. The electrostatic attractions even enhance the protein aggregation due to BLG-BLG interaction (Majhi et al. 2006). An association between peptides and BLG due to electrostatic attraction was recently observed in the study of Kusters et al. (2013). They have also observed stronger binding or even the formation of a covalent interaction between the free sulfhydryl group of the peptides and a cysteine residue of the BLG.

The three-dimensional structure of a protein may facilitate interactions with other components found in foods or the gastrointestinal (GI) tract lumen, thus altering their stability to proteolysis which is related with gastric digestion (Moreno, Mackie, and Mills 2005). It has been also reported that the effects of physiological surfactants on the simulated GI proteolysis of bovine BLG, showing that under certain conditions BLG is almost completely protected from simulated digestion (Mandalari et al. 2009).

BLG is critical protein for the nutritional intake during digestion, as well as for a successful manipulation of the physical and sensorial attributes of colloidal food systems (such as emulsion stability, creaminess and rate of flavor release). However, both emulsions and saliva are complex systems. For instance, human saliva consists mainly of water (99.5%), various proteins (0.3%), small organic compounds and inorganic salts and has a pH of around 6.8 (Zalewska et al. 2000). Therefore, many scientists preferred to focus on the major protein component of saliva, highly-glycosylated mucin, and its interaction with various food proteins used as emulsifier in order to understand protein-protein interaction mechanisms and the effect of protein charge on the emulsion-saliva interaction. Thus, one of the most commonly used proteins to investigate the interaction with saliva/mucin is BLG.

### *Saliva interaction with BLG*

Kelly et al. (2010) studied the interaction between BLG and salivary proteins and investigated the effects of protein concentration on astringency. Due to the high number of samples, they divided sensory evaluation into three studies. The first study investigated the effect of protein concentrations ranging from 0.25 to 4%. Protein concentrations in the second study ranged from 4 to 13%. In the third study, samples were selected to cover the whole range of concentrations, and time-intensity analysis was chosen to provide more comprehensive data (i.e., time to reach maximum astringency, astringency duration). They found that the changes in the astringency by varying the concentration of protein are dependent on pH. At pH 3.5, astringency was significantly increased in the protein concentration range from 0.25 to 4% (wt/wt) and then remained constant from 4 to 13% (wt/wt). In contrast, at pH 2.6, astringency was decreased with an increase in protein concentration [0.5–10% (wt/wt)]. This suggests a complex relationship that includes pH and buffering capacity of the beverages. Furthermore, saliva flow rates increased with increasing protein concentrations, showing that the physiological conditions in the mouth changed with the protein concentration. Maximum turbidity of whey protein–saliva mixtures was observed between pH 4.6 and 5.2. Both sensory evaluation and *in vitro* study of the interactions between BLG and saliva indicate that the astringency of whey proteins is a complex process determined by the extent of aggregation occurring in the mouth, which depends on the pH of the whey protein beverage and buffering capacity in addition to the flow rate of the saliva. This trend was not same with the study of Beecher et al. (2008) in which maximum astringency was found at pH 3.4 and a decrease in astringency was observed when the pH was lowered to 2.6. The difference may be due to the type of protein used and the processing parameters. Beecher et al. (2008) used whey protein isolates (6% (wt/vol) protein) and heated the whey protein beverages. Meanwhile, Kelly et al. (2010) used BLG because of its higher purity and the samples were unheated to reduce the degree of protein aggregation. These factors may have contributed to the different results.

Peak turbidities resulting from polyphenols and mucin interactions were correlated with maximum astringency (Monteleone et al. 2004; Condelli et al. 2006). Nevertheless, such a linear correlation with protein-based astringency was not observed by Kelly et al. (2010), who explained this by the differences between intermolecular binding mechanisms of proteins and polyphenols. Interactions between polyphenols and salivary proteins are a result of hydrogen bonding (Haslam 1974) or hydrophobic interactions (Hagerman and Butler 1980). These interactions have minor pH dependence. In contrast, as protein charge is pH dependent, interactions between saliva and whey proteins will be also pH dependent. Thus, Kelly et al. (2010) suggested a dynamic model to explain the astringency of proteins as pH would depend on the amounts and buffering capacities of saliva and protein beverage, coupled with changes in saliva flow.

Vardhanabhuti et al. (2011) investigated the perception of astringency in relation to the effect of BLG at pH 3.5 and 7.0 by studying the lubrication properties of saliva using a tribological approach. They have used fresh stimulated whole saliva without any treatment in all experiments. Saliva was adsorbed onto surfaces of a rotating poly(dimethylsiloxane) (PDMS) ball and disc to form a film under conditions that mimic the rubbing contacts in the oral cavity (Bongaerts et al. 2007) and the lubricity of saliva films upon exposure to astringent compounds was measured. While addition of non-astringent BLG at pH 7.0 slowly increased the friction of saliva films between tribopair surfaces, BLG at pH 3.5 rapidly increased the friction coefficients of saliva, similar to other astringent compounds (e.g. epigallocatechin gallate and alum). This supports the hypothesis that astringency of BLG is closely associated with the loss of lubricating properties of saliva, which is in agreement with the well-accepted astringency model of polyphenols. They concluded that aggregation and/or precipitation of whey proteins at their pI's, as well as charged interactions between whey proteins and salivary proteins, can contribute to the astringency of whey proteins at low pH. A number of studies already provided evidences that support the role of electrostatic interactions in milk protein astringency (Malone, Appelqvist, and Norton 2003; Vardhanabhuti and Foegeding 2010; Vardhanabhuti et al. 2010). An outstanding significance of the study by Vardhanabhuti et al. (2011) is that it showed that these interactions lead to the loss of lubricating properties of saliva. Increasing BLG concentration at pH 3.5 (0.5–10% w/w) caused a rapid increase in friction coefficient; however, at the highest protein concentration, the friction coefficient was lower than the values observed for the lower protein concentrations, although it was still higher than the values observed from water. This clearly supports the hypothesis that precipitation of whey proteins from mixing with saliva or interaction between positively charged whey proteins and salivary proteins leads to an increase in friction and/or the loss of saliva lubrication, and thus contributes to astringency of whey proteins at low pH. Additionally, Vardhanabhuti et al. (2011) suggested that static tribology testing is different from the dynamic in-mouth system such that a simple relationship between friction and sensory

astriogeneity cannot be found for all conditions. This is due to the complexity of the dynamic saliva model and whey proteins, in addition to the variety of parameters such as concentration, pH, heat, and surface properties that effect the friction. Overall, using tribology to study the lubrication properties between saliva or saliva proteins with food proteins is a valuable approach to study the perception of astringency.

### **Mucin interaction with BLG**

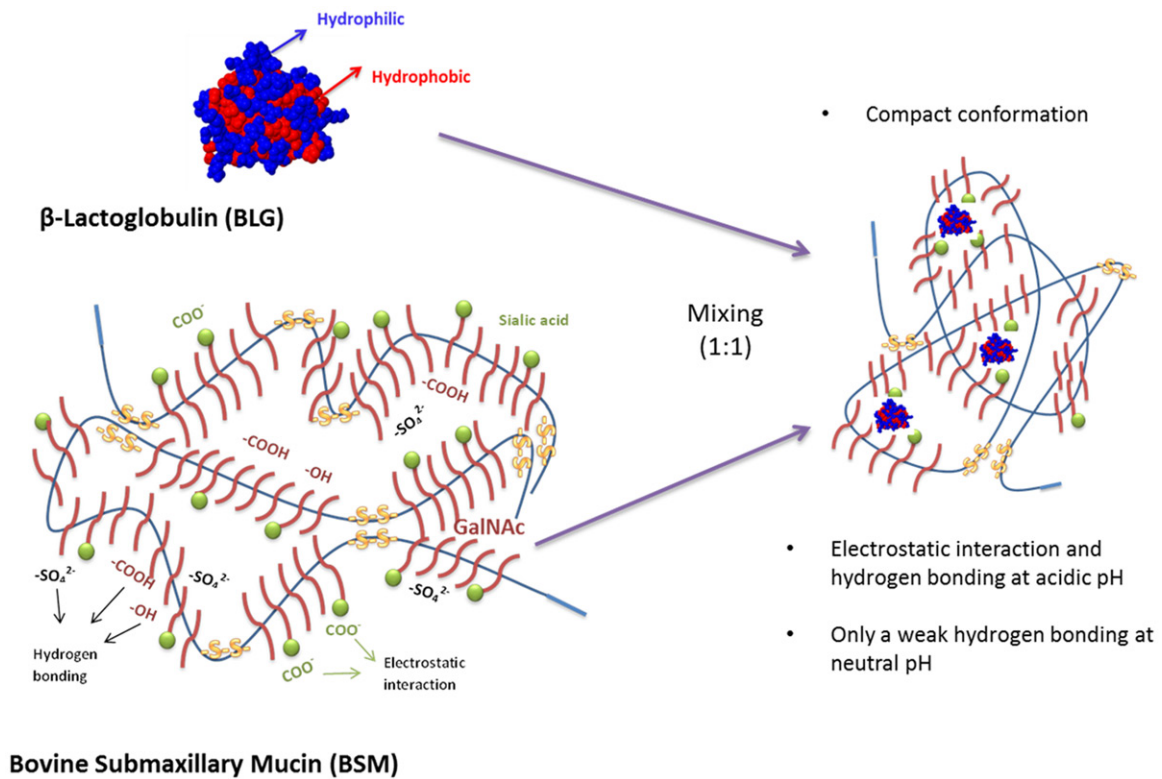
Recently, our group has focused on the interactions of bovine submaxillary mucin (BSM) with BLG at different pH values in order to understand the protein-protein interactions in a food-saliva model system. Among several mucin types involved, submaxillary mucin is the one most related to oral processing. In order to establish a basis to understand food oral processing of the two proteins on the molecular level, the ratio of 1:1 was selected. Dynamic light scattering (DLS) was employed to investigate the changes in hydrodynamic radius of proteins. High and low field Nuclear Magnetic Resonance (NMR) techniques were employed to characterize the interaction by monitoring changes on the chemical shifts of proteins residues. Finally, secondary and tertiary structures of proteins were studied using circular dichroism (CD) spectroscopy (Çelebioğlu et al. 2015). The zeta potentials of the proteins showed that the surface charge characteristics of the BLG-BSM mixture were dominated by the BSM. Results from DLS measurements suggested that the interaction between the proteins caused a more compact conformation of BSM as the BLG integrated into the BSM. Far UV and near UV CD spectroscopy studies of the mixture of the two proteins showed intermediate spectra compared to each protein alone for both secondary and tertiary structure of the proteins, which may support the proposed interaction between them. High field NMR measurements are consistent with polar interactions at pH 5 and pH 3, whereas no significant interaction was detected at pH 7.4. Longer  $T_2$  relation times were observed for the BLG-BSM mixture, especially at acidic pH, which was attributed to higher water mobility. The higher water mobility may indicate that fewer charged groups on the proteins surfaces were available for protein-water interaction due to the interactions between the hydrophilic parts of BLG and BSM compared to BSM alone. The overall conclusion is that the higher hydrophilic interaction between the proteins at lower pH supported the pH dependent activity of both BLG and BSM. Furthermore, the positively charged groups of BLG, especially at acidic pHs, neutralized negatively charged groups of BSM and caused the BSM to coil or contract into a smaller hydrodynamic volume (Figure 4), as suggested by Shrivastava and Nair (2003) as well. In fact, even a weak hydrogen bonding between BLG and BSM brings about aggregation of mucins into a more compact structure at pH 7.4. The NMR results also implied that negatively charged BLG has a tendency to interact with negatively charged mucin via secondary interactions (hydrogen bonding and hydrophobic effects), where the

electrostatic interactions are unlikely to be the main reason of the binding.

Since BSM and BLG showed an interesting interaction, the interfacial properties of BLG, BSM, and their mixtures at air/liquid interface also draw attention. It is important to understand the interfacial rheological properties of adsorbed BLG and mucin layers and their network formation at the liquid surface that are relevant in a wide range of applications such as foam and emulsion stability or multiphase fluids processing. The interfacial rheological properties of solutions of BLG (as a model food compound) with a salivary mucin protein, BSM, at different pHs, were investigated in another recent study by the authors (Çelebioğlu et al. 2017). The results showed that all protein layers (BSM, BLG, and BLG-BSM mixtures) formed at air/water interface has some similarities such as a rapidly developed elastic interfacial network, low frequency dependence of the interfacial modulus. The high molecular weight BSM formed a weak viscoelastic interfacial network (lower modulus) compared to BLG at all pHs, which is destroyed even at low strain (0.003%). The pH has a significant effect on the surface density of adsorbed BLG proteins, as it determines the net charges and the modulus of the interfacial network. At pH close to pI, electrostatic repulsions between the adsorbed BLG molecules are minimized at the interface, and it promotes the formation of stable adsorbed layer with a high elastic modulus. Furthermore, BLG molecules move faster due to their smaller size/mass than mucins, and dominate the surface adsorption and the network formation for the BLG-BSM mixtures. However, BLG-BSM protein mixtures exhibited interfacial properties with lower elastic and viscous moduli than BLG, as a result of competitive displacement of BLG proteins with BSM molecules from the interface. We propose that BSMs decreased the surface viscoelasticity and the rigidity of the BLG layers through the penetration of the hydrophobic parts of BSM between the adsorbed BLG molecules and disrupt their cohesive assembly, which was most pronounced at pH 5 (Figure 5). Moreover, it is important to stress that facile attraction of BSM molecules towards BLG layer within water phase is not sufficient to activate this mechanism. At pH 3, for example, despite electrostatic attraction between oppositely charged BSMs and BLG layer, the reduction in viscoelasticity and rigidity is weaker compared to that at pH 5. This can be explained by that overall hydrophilic nature of the interaction between them hinders the hydrophobic parts of BSM to disrupt the assembled layer of BLG and extend its interaction with air.

We have also studied the surface adsorption of BLG, BSM and their mixture at the solid/liquid interface (Çelebioğlu et al. 2016). The study based on bicinehoninic acid (BCA) protein qualification assay showed that mucins were not only higher than BLG in the adsorbed amount (masses) onto the solid hydrophobic surface, but also adsorb in a more compact conformation due to a high flexibility to accommodate themselves in a narrow space and/or a possibility to form multilayers (Çelebioğlu et al. 2016). However, BLG can readily dominate the initial stage of surface adsorption at the solid/water interface in the mixture solution of





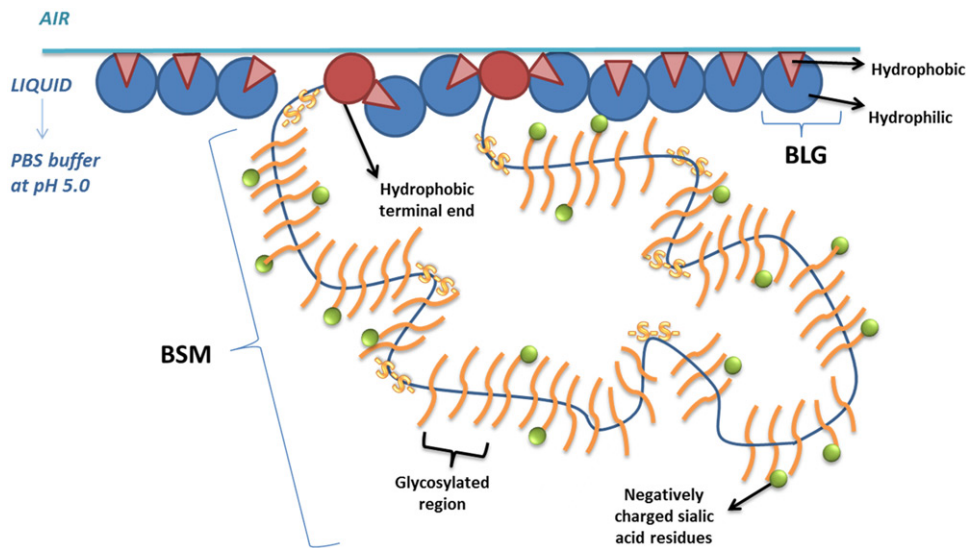
**Figure 4.** Illustration of the interaction between  $\beta$ -lactoglobulin and bovine submaxillary mucin. (Çelebioğlu et al. 2015).

BSM and BLG due to the ability of the smaller and lighter BLG molecules to reach the surface faster than BSM. In the adsorption of the BLG-BSM mixture onto hydrophobic solid surfaces, it was assumed that there is a large portion of “free” BLG molecules in the mixed protein solutions, and that they participate in the surface adsorption process in competition with BSM. In addition to BSM, PGM was also used for interaction studies with BLG. The fact that both mucins are highly relevant to food digestion process, yet to different organs, is the first motivation to compare them. Additionally, in parallel with common structural features of the two mucins (Bansil, Stanley, and LaMont 1995; Sandberg, Blom, and Caldwell 2009), reported differences in their biophysical properties, especially the lubricating properties (Lee et al. 2005; Nikogeorgos, Madsen, and Lee 2014), may lead to different interaction with BLG and alteration in the lubricating properties. The surface adsorption results showed that the net effect of mixing BLG and PGM is featured with substantially decreased adsorbed masses compared to the neat PGM solutions. Thus, it was suggested that BLG dominates also the solid/water interface for BLG-PGM mixture.

Another novel approach was to apply tribological techniques to investigate the interaction of BLG with mucins and investigate how it affects their lubricating properties (Çelebioğlu et al. 2016). Recently, tribology has emerged as a new instrumental approach to investigate oral processing of food emulsions in simulated oral environment (Meyer et al. 2011; Vardhanabhuti et al. 2011; Chojnicka-Paszun, de Jongh, and de Kruif 2012; Chen and Stokes, 2012; Van Aken, 2013; Selway and Stokes, 2013; Prakash, Tan, and

Chen 2013; Chen, Liu, and Prakash 2014; Joyner Melito, Pernell, and Daubert 2014). In turn, this is often correlated with food’s sensory perception (Meyer et al. 2011; Vardhanabhuti et al. 2011; Chojnicka-Paszun, de Jongh, and de Kruif 2012; Selway and Stokes 2013; Prakash, Tan, and Chen 2013). Tribology is particularly useful for understanding the behavior of thin films formed between two opposing surfaces where rheological and structural/mechanical properties of food may no longer explain their behavior sufficiently. Recent applications of tribological techniques allowed for quantitative characterization of the lubricating properties of the fluids involving saliva and BLG or other astringents. For example, Vardhanabhuti et al. (2011) showed that addition of BLG into a soft tribological interface increased the interfacial friction forces, yet at varying rates depending on pH. Aggregation of macromolecules (BLG) with hydrogel (saliva) is, however, a complex process influenced by a number of parameters.

Recently, we have investigated the molecular-level interaction between mucins and BLG by means of tribological approaches according to mucin type, solution pH, and protein concentration. Hydrophobic interfaces, namely PDMS-PDMS and polyoxymethylene (POM)-PDMS, were employed for feasible adsorption of the proteins and consequent possibility of assessment of the boundary lubricating properties. The structural and mechanical properties of thin films generated from two types of mucins, namely, BSM and PGM in aqueous environment, were also investigated by Madsen et al. (2016) who showed that both mucins generated hydrated films on hydrophobic PDMS surfaces from



**Figure 5.** Interfacial layer of BLG-BSM mixture showing the penetration of the hydrophobic parts of BSM between the adsorbed BLG molecules and disorder their cohesive assembly, which is most pronounced at pH 5. (Çelebioğlu et al. 2017).

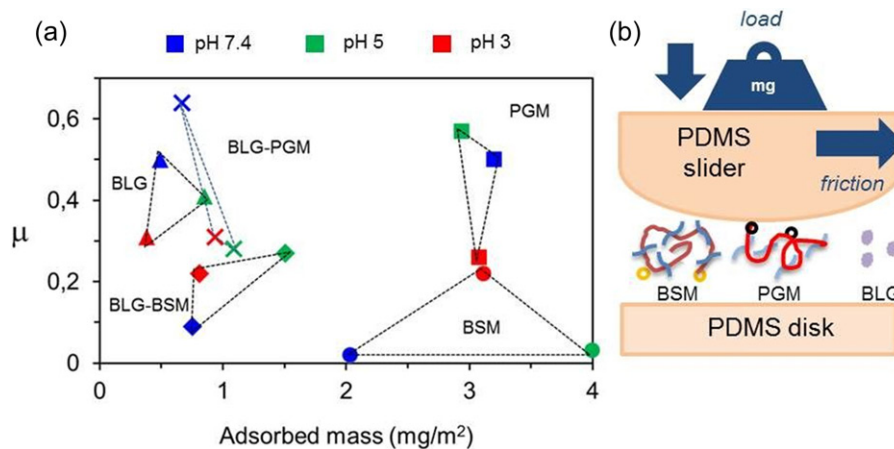
spontaneous adsorption arising from their amphiphilic characteristic.

Overall, we have observed that mucins have effective lubricating properties, in particular BSM, compared to BLG. Nevertheless, nearly ignorable lubricating effect by PGM, despite its facile surface adsorption, suggests that other parameters than adsorbed masses play a significant role to impart superior lubricity of BSM to PGM or BLG (Figure 6). While both pin-on-disk tribometry and mini-traction machine (MTM) were employed to provide the tribological contacts with different contact pressure, speed range, and slide/roll ratio, the dominating lubrication mechanism by the protein solution was boundary lubrication. Surface adsorption and lubricating properties of mixed protein solutions, such as BLG-BSM and BLG-PGM, with respect to neat protein solutions were of prime interest as it can be compared with the well-known role of BLG as astringency to form a complex with saliva and rapidly deplete from the tribological interface at acidic pH (3.5, for example). Even in the absence of tribostress, the adsorbed masses of the mixed protein solutions reduced significantly, and BLG appeared to dominate the surface adsorption event, presumably due to the reduced concentration of mucins as well as the Vroman effect. Nevertheless, excellent lubricity was still observed at pH 7.4 and BSM apparently dominated the tribological interface, which highlights the excellent lubricating capabilities of BSM. Although being still relatively more lubricious than the other proteins, the BLG-BSM mixture showed the highest level of degradation in the lubricity of BSM at pH 5, which contrasts the case of BLG-saliva interaction. This is due to that instead of strong aggregation, as in BLG-saliva, the lubricating properties of BLG-BSM are determined by competitive adsorption of the two proteins onto substrates. Most importantly, these observations further suggest that BLG and BSM molecules do not form strong aggregates, especially under tribological stress. PGM's intrinsically weaker lubricity remained largely unchanged even in the interaction with BLG.

Consequently, mucins and BLG showed an interesting interaction and different surface adsorption behavior at solid/liquid or liquid/air interfaces. However, little has been known about the hydrophobic interaction between the BLG and mucins, and the role of hydrophobic residues of these proteins to date.

### **Mucin/saliva interaction with whey protein stabilized emulsion**

Due to their emulsifying property, proteins found in milk or in other foods frequently used in food-emulsions-systems. Thus, studies of the mucin/saliva interactions with well-ordered protein stabilized emulsions are very close to defining the behavior of real food products. An understanding of the oral processing of an emulsion is critical for a successful manipulation of the physical and sensorial attributes of colloidal food systems, such as emulsion stability, creaminess and rate of flavor release. Food emulsions are exposed to a range of processing steps during oral consumption such as mixing with saliva, heating or cooling to the body temperature. In addition, air is introduced, and the emulsions come into contact with oral surfaces and are exposed to complicated saliva flow profiles. It is anticipated that saliva-emulsion interactions play an important role in understanding emulsion perception, however, little is known about the oral behavior of food emulsions (van Aken et al. 2007). Previous studies dealing with the interaction between emulsions and saliva was focused on, firstly, sensory analysis of emulsions in relation to the saliva flow (Engelen et al. 2003) and composition (Engelen et al. 2007), and secondly on the physicochemical properties of emulsions *in vitro* (Barylko-Pikielna, Martin, and Mela 1994; Kilcast and Clegg, 2002; Metcalf and Vickers 2002; de Wijk et al. 2003). Then, the flavor release from emulsions (de Roos, 2003) as measured in mouth models (Doyen et al. 2001; van Ruth, King, and Giannouli 2002; van Ruth and Roozen, 2000a, 2000b) and *in vivo* via



**Figure 6.** (a) Coefficient of friction,  $\mu$  (PDMS-PDMS pair) vs. adsorbed mass,  $\Gamma$  (PDMS surface) of the protein solutions (1 mg/mL concentrations only). The color codes are for different pHs: red for pH =3, green for pH =5, and blue for pH =7.4. Different symbols represent different protein or mixed protein solutions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article. (Çelebioğlu et al. 2016)). (b) Schematic presentation of protein molecules under tribological stress using pin-on-disk tribometer with PDMS-PDMS tribopair.

e.g. electronic noses (Miettinen et al. 2002) are other subjects of studies.

van Aken et al. (2003) reported a strong aggregating effect of saliva on both proteins (milk protein – including caseinate and whey protein) and surfactant-stabilized emulsions. In addition, explorative experiments were carried out to gain insight into the oral behavior of commercial emulsions (van Aken et al. 2007). They have further investigated the aggregation phenomena to elucidate the physicochemical effects of saliva on protein-stabilized food emulsions, with an emphasis on the role of high molecular weight mucins. They observed that mixing emulsions with saliva or mucin can induce droplet aggregation and suggested that the observed behavior affects the texture and rheological properties of emulsions and thus the sensory perception of emulsions. For instance, saliva-induced droplet flocculation leads to an increase in low-shear viscosity, which is related to product viscosity with mouthfeel (van Aken et al. 2007).

The high molecular weight salivary mucin was also modeled by porcine gastric mucin (PGM), which consists of both MUC5AC and MUC6 (Nordman et al. 2002). MUC5AC has particularly large similarities to the high molecular weight human salivary mucins (MUC5B) (van Klinken et al. 1998; Offner et al. 1998). In the study of Vingerhoeds et al. (2005), the rheological behavior of whey protein isolate (WPI) emulsions upon addition of PGM was determined. Aggregation of the emulsions was clearly observed and the results suggested that mucin, as the main component in saliva, is responsible for the observed aggregation, but also indicated that other salivary components could play a role. They have observed a reversible aggregation upon dilution with water, indicating that the bonds between the emulsion droplets were relatively weak and coalescence was negligible. Hence, they concluded that the aggregation might not be due to covalent bonds or to a precipitation mechanism such as an antigenic reaction. The particle size of the emulsions was not altered by the addition of PGM, meaning that droplet flocculation can arise from depletion or a bridging mechanism (Blijdenstein, Hendriks et al. 2003;

Blijdenstein, van Vliet et al. 2003; Dickinson and Pawlowsky 1997). The mechanism of droplet flocculation was determined by the nature of the interaction between the surfactants and the polymers. For instance, in the absence of the interaction between surfactants and polymers, emulsion droplets can lead to depletion flocculation. On the other hand, under attractive interactions between them, emulsion droplets can impose bridging flocculation (Healy and La Mer, 1964). To elucidate the mechanism behind the observed mucin-induced aggregation, Vingerhoeds et al. (2005) performed de-mixing experiments as a function of the concentration of PGM. Removal of excess BLG by washing the emulsion before mixing with PGM had no effect on the occurrence of flocculation as observed by light microscopy. This indicates that the dissolved protein emulsifier is not involved in the flocculation. Overall, those observations are indicative of that depletion flocculation is the leading mechanism in the case of mucin/saliva interaction with protein-stabilized emulsion.

#### **Mucin/saliva interactions with BLG-stabilized emulsion**

Since BLG dominates the total whey protein content (>50%) and has good emulsifying properties, most studies have focused on mucin/saliva interactions with BLG-stabilized emulsions. Using this approach a simplified model of oral processing of real foods with saliva can be established. Various attempts have been reported in literature to explore the effect of mixing saliva/mucin with the milk proteins, especially by using the BLG-stabilized emulsions (Sarkar et al. 2009; Sarkar, Goh, and Singh 2010; Silletti et al. 2007; Vingerhoeds et al. 2005). Sarkar et al. (2009) determined the physicochemical effects of the porcine gastric mucin (Sigma, Type II) on negatively charged BLG-stabilized emulsion. They showed that the BLG-stabilized emulsions remained homogeneous in the presence of mucin at low concentrations. However, the emulsions showed network-like flocculation by addition of mucin at the concentration of 1.0 wt%. These emulsion–saliva mixtures (containing added 1.0 wt%

mucin) reverted back to homogenous dispersions after dilution with water, which indicated that the flocculation was reversible. Moreover, Sarkar et al. (2009) mentioned that for the BLG-coated droplets, the presence of some positive patches along the adsorbed BLG molecules probably resulted in electrostatic interactions with the negatively charged mucin molecules. Moreover, there is also possibility of hydrophobic interaction between unfolded BLG and mucin molecules at the adsorbed layer at low ionic strengths of artificial saliva.

Silletti et al. (2007) also hypothesized that depletion flocculation was responsible for the observed flocculation. To further unravel the mechanism, they investigated the role of electrostatics on the behavior of emulsion/saliva mixtures. Emulsions stabilized with differently charged surfactants and proteins were mixed with saliva. Strongly negatively charged emulsions (SDS and Panodan) did not flocculate, and this could be due to the electrostatic repulsion between the droplets overcoming the attractive depletion and van der Waals interactions. However, reversible flocculation was observed for neutral and weakly negatively charged emulsions (Tween 20 and BLG pH 6.7). Silletti et al. (2007) suggested that this is probably due to depletion interactions, induced by large salivary protein like mucins, in combination with the van der Waals interactions and the sufficiently low electrostatic repulsion between the droplets. Positively charged emulsions (e.g. CTAB, lysozyme and BLG at pH 3.0) showed irreversible flocculation, leading to rapid phase separation. These findings point to a role of electrostatic attraction between the negatively charged proteins present in saliva and the positively charged surfaces of the emulsion droplets. Certainly, these results indicate that the sign and the density of the charge on the surface of the droplets contribute significantly to the behavior of an emulsion when mixed with saliva. Depending on the charge, saliva-induced emulsion flocculation is driven by two different main mechanisms: depletion flocculation and electrostatic attraction.

By employing mucin and BLG-stabilized emulsions, Silletti et al. (2010) investigated the interactions between BLG-stabilized emulsion droplets and salivary proteins (mucin), by means of different techniques, e.g. infrared spectroscopy, Western blotting, PAS staining and SDS-PAGE coupled to MS. They showed that adsorption/association of mucin onto the emulsion droplets is related to the type of emulsifying proteins at the oil-water interfaces and it is driven by the overall net charge at the droplet's oil-water interfaces, i.e. negative for BLG-stabilized emulsion at neutral pH. Sarkar, Goh, and Singh (2010) also mentioned that the presence of a low level of mucin appears to promote the flocculation of BLG-stabilized emulsions, possibly through a bridging mechanism. Broadly, neutral and negatively charged emulsions undergo reversible depletion flocculation whereas cationic emulsions show irreversible associative electrostatic interactions with mucin and salivary salts. Similarly, Teng et al. (2016) found that cationic polymers interact readily with mucin and exhibited better mucoadhesion properties than unmodified BLG. Moreover, Singh and Ye (2013) showed depletion flocculation of a BLG-stabilized

emulsion (20 wt% soybean oil, 1.0 wt% BLG) when mixed with an artificial saliva composition containing PGM. This flocculation was largely dependent on the mucin concentration (a critical mucin concentration of 0.4 wt%) present in the saliva. Thus, the differences found between negatively charged and cationic emulsions signify the importance of the surface charges on the interaction/flocculation observed in the presence of saliva/mucin. Moreover, the effect of mucin concentration on emulsion flocculation (of similar-surface-charged-emulsions) may also further indicate the importance of surface hydrophobic interaction.

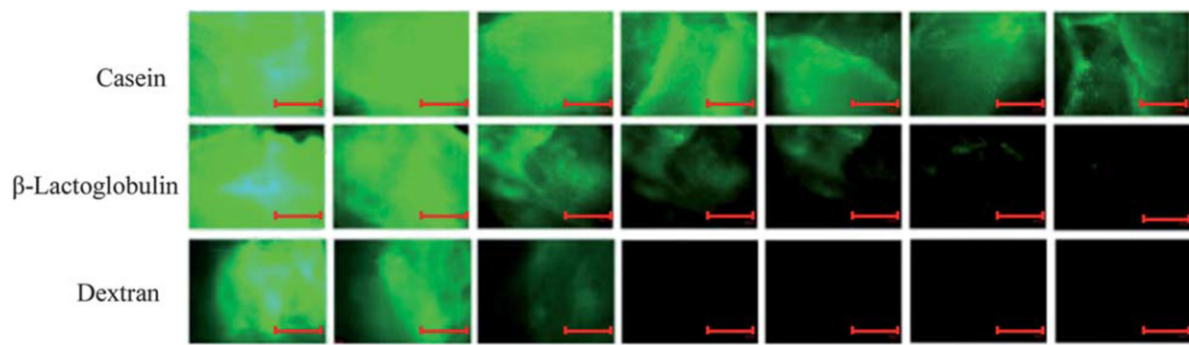
## Mucin/saliva interaction with non-whey proteins

### Mucin/saliva interaction with casein

Caseins evolve from members of a group of secreted calcium (phosphate)-binding phosphoproteins (Kawasaki and Weiss, 2003; Rijnkels et al. 2003; Kawasaki, Suzuki, and Weiss 2004; Kawasaki, Lafont, and Sire 2011; Lemay et al. 2009). In eutherian milks, at least three and normally four gene products of casein (CN) are found; namely,  $\alpha$ S1-,  $\alpha$ S2-,  $\beta$ -, and  $\kappa$ -CN, but in some species two quite different  $\alpha$ S2-CN-like genes are active, raising the total number of gene products to as many as five. (Holt et al. 2013).

Caseins constitute about 80% of the proteins of bovine milk and they contain high numbers of proline residues evenly distributed throughout their amino acid sequences and have relatively open structural features (like the salivary proline-rich proteins). For this reason, isolated caseins have often been used as model proteins in various polyphenol-protein studies (Jöbstl et al. 2004; Pascal et al. 2008; Yan, Hu, and Yao 2009).

Withers et al. (2013) recently investigated the potential adhesion of milk proteins to porcine oral mucosa in vitro. This study aimed to evaluate the retention of milk proteins (casein and BLG) on the oral epithelial surfaces including buccal and tongue tissues. The hypothesis of this study was that the adhesion of milk proteins to the oral epithelium may be related to the mouth drying in dairy products. Purified casein and BLG were fluorescently-labeled, placed on porcine oral mucosal tissues and their resistance to wash out with simulated saliva was monitored using fluorescence microscopy (Figure 7). Fluorescein isothiocyanate (FITC)-dextran was used as a negative control in these experiments because it is not expected to provide substantial retention effects as a nonionic, poorly mucoadhesive polymer. Unlabeled samples showed no fluorescence at this wavelength, indicating that no intrinsic fluorescence would be demonstrated by the proteins alone. Therefore, all fluorescence observed originated from the FITC-bound proteins with casein exhibiting greater fluorescence than BLG in this study. Dynamic light scattering indicated that the labeling process did not destroy the micellar structure of casein in solution. The presence of BLG on the mucosal surface was detected even after 30 washes, which confirms its stronger ability to adhere to buccal membrane compared to FITC-dextran. Thus, Withers et al. (2013) suggested that although BLG interacted with the cheek mucosal surface, the salivary



**Figure 7.** Exemplary fluorescent microphotographs, showing the retention of casein,  $\beta$ -lactoglobulin and dextran on buccal mucosa against the number of washes with artificial saliva. Scale bars indicate 500  $\mu$ m at image magnification (Withers et al. 2013).

washes were able to remove this protein eventually. Casein was found to remain on the mucosal surface in substantial quantities even after 50 washes, indicating that this protein also strongly interacts with mucosal surface. Moreover, thiol content measurements showed that the level of thiols in BLG was found to be significantly higher than in casein ( $p < 0.001$ ). Both casein and BLG were found to be negatively charged at pH 9.0 as expected. The greater zeta-potential of BLG implies a greater stability of the protein in solution, relative to casein. On the other hand, the viscosity of casein solution was 2.3 times higher than BLG, which is likely related to the ability of casein to form naturally self-assembled micellar structures (Hägerström and Edsman, 2003). Overall, the results supported that the interaction between mucin present in the saliva with BLG, but not with casein. This may be a result of the markedly different rheological properties of casein, in part a result of its micellar nature (Withers et al. 2013). In addition, Withers et al. (2013) proposed that electrostatic interactions are unlikely to be the main cause of mucoadhesion because both proteins are overall negatively charged under the near-neutral pH of the oral cavity. However, compared to BLG, the less negatively charged casein should feel a weaker repulsion from the negatively charged mucosal surface.

Another recent study by Withers et al. (2014) aimed to test the hypothesis that casein within protein-fortified beverages at neutral pH could contribute to perceived mouth drying. Sequential profiling found that the whey protein concentrate (WPC75; containing 75% wt/wt protein) and sodium caseinate (NaCas; containing 85% wt/wt protein) samples were not significantly different for mouth drying or chalkiness. Previously, it was suggested by several authors that mouth drying was an attribute found to build substantially during the consumption of oral nutritional supplement (ONS) dairy-based beverages, and it could originate from whey or casein sources (Methven et al. 2010). Previously, whey-based dairy beverages have found to be astringent, especially at reduced pHs; it has been proposed that the negatively charged salivary proteins bind electrostatically to positively charged whey proteins to precipitate around the oral cavity (Sano et al. 2005; Ye, Streicher, and Singh 2011) or bind to oral epithelial cells (Ye et al. 2012). Similarly, Withers et al. (2014) found that ONS mouth drying appears to originate predominantly from whey proteins, and that

masking mouth drying is not a simple task. However, the potential for casein to contribute to mouth drying was also demonstrated. Whether casein-derived mouth drying is due to the presence of  $\gamma$ -CN remains unanswered, and further investigation, including the use of HPLC to quantify  $\gamma$ -CN in heat-treated, protein-fortified beverages over shelf life, was recommended.

In a different point of view, Huq et al. (2016) also studied the interaction between casein and saliva using adhesion, SDS-PAGE, and ELISA techniques. Their aim was to demonstrate the repair of early dental caries lesions by the application of the remineralisation technology based on casein phosphopeptide-stabilized amorphous calcium phosphate complexes (CPP-ACP). Huq et al. (2016) showed that the two predominant peptides of the casein phosphopeptide (CPP) have the ability to interact with selected salivary proteins and peptides found as components of the enamel pellicle. The sequences of both  $\alpha$ S1-CN (59–79) and  $\beta$ -CN (1–25) peptides have hydrophobic and hydrophilic regions. This enables them to bind to a range of proteins through hydrophobic or electrostatic interactions. Nevertheless, it is evident that the CPP are not promiscuous binders of salivary proteins, but display selectivity (Huq et al. 2016). After all, they attributed the non-covalent interactions between specific salivary proteins and peptides and the CPP, to the concomitant retention of mineral ions and peptides derived from the CPP-ACP complexes within plaque on the enamel surface. In summary, Huq et al. (2016) elucidated the mechanism of anticariogenicity displayed by the CPP-ACP nano-complexes involving several molecular interactions between organic and inorganic molecules. These interactions include the binding of the CPP to specific salivary proteins and peptides.

Overall, the above studies propose that casein attracts the salivary proteins only via non-covalent interactions due to its naturally self-assembled micellar structure. The binding of casein to salivary proteins is weak, however, some predominant peptides of casein display selective binding. Moreover, casein is able to interact with mucosal surface and this interaction is stronger than the casein-mucin interaction. Nevertheless, different species of casein or different casein complexes may show different mucoadhesion and interaction properties with the salivary proteins. It is to note that in contrast, BLG shows significant hydrogen binding

and hydrophobic attraction to mucin while the electrostatic attraction is less pronounced. Hence, further studies are needed in order to assess the interaction mechanisms of casein with salivary proteins, by using different casein species with different charges and protein content, with variable mucosal surfaces.

### **Mucin/saliva interaction with gelatin**

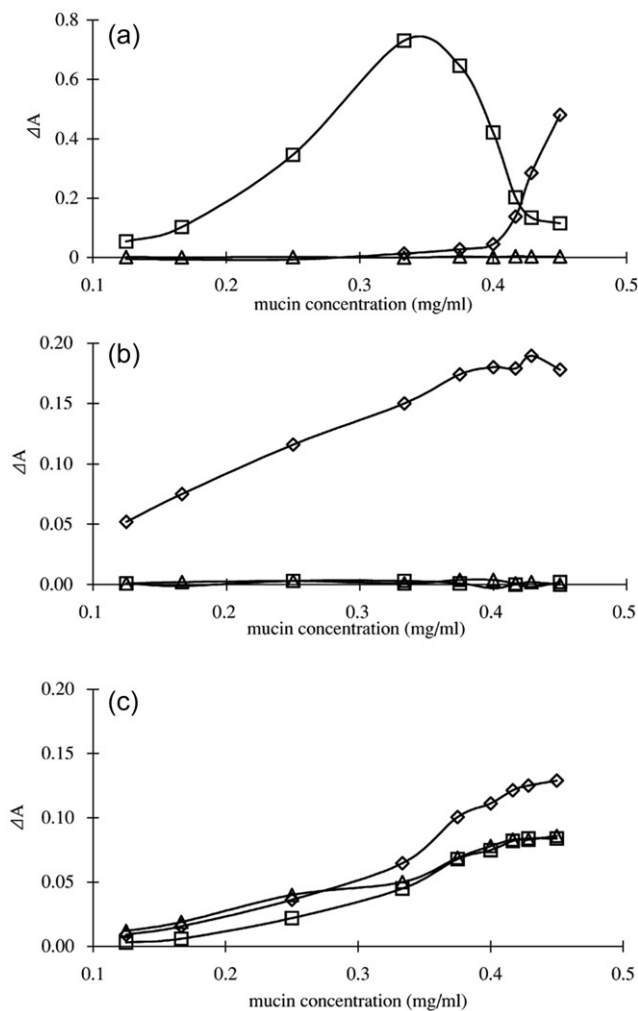
Gelatin, one of the most commonly used hydrocolloids in the food products, is commercially derived from collagen extracted from the bone, skin or tendons of animals. It contains glycine, proline and hydroxyproline, and is used as a thickening agent in dessert jellies, confectionery jellies and gums (Johnston-Banks, 1990). Gelatin is also used as mucoadhesive materials due to its ability to adhere to the mucus layer and release the loaded drug in a sustained manner. Wang et al. (2000) demonstrated that aminated gelatin microspheres, with high primary amino group content, showed a sustained amoxicillin release characteristic and a considerable gastric mucoadhesive property *in vitro*. Mucoadhesion involves different kinds of interaction forces between mucoadhesive materials and mucus surface, such as electrostatic attraction, hydrogen bonding, van der Waals forces and mechanical interpenetration and entanglement. Of the *in vitro* test, commercial mucin is frequently used as a substitute for fresh mucin because of its reproducible quality and easy availability, although it does not possess the same viscoelastic properties as freshly isolated mucus gel (Madsen, Eberth, and Smart 1996). Nevertheless, it is still proved to be effective to provide preliminary information about the interaction with mucoadhesive materials (He, Davis, and Illum 1998; Rossi et al. 2000).

Wang et al. (2001) utilized two kinds of commonly used commercial mucins with different content of sialic acid (Sigma mucin type III from porcine stomach – 1% sialic acid and mucin type I-S from porcine pancreas – 12% sialic acid) to evaluate their interaction with gelatin by measuring absorbance via spectrophotometer. They showed that the interaction between gelatin and mucin changed dramatically in varying environmental conditions. In purified water, gelatin (IEP = 9.0) is positively charged, and a strong interaction with mucin was observed as a result of the electrostatic attraction between them under this condition. However, aminated gelatin, with a higher positive charge density (higher amino group content) than gelatin, showed a lower interaction with mucin at a low mucin concentration. This could be due to higher solubility of aminated gelatin in water than the gelatin. Moreover, Wang et al. (2001) observed a strong interaction at a higher mucin concentration even when the aminated gelatin concentration was relatively very low. In contrast, gelatin (IEP = 5.0) did not show any interaction with mucin in purified water because it carried a net negative charge as mucin did under this condition. In the case of PBS, gelatin (IEP = 9.0), as well as gelatin (IEP = 5.0), showed no interaction with mucin, although gelatin (IEP = 9.0) was expected to reveal an electrostatic attraction with mucin as in the case of purified

water (Figure 8) (Wang et al. et al. 2001). The results for gelatin were ascribed to the unfavorable biopolymer conformation in the presence of high electrolyte concentration where the gelatin was dehydrated, thus shielding the free amino group content and reducing the available positive charge. Furthermore, due to the existent electrolyte, the ionization of sialic acid residues of mucin was greatly reduced, the electrostatic attraction between mucin and gelatins decreased, hence resulting in a weakened interaction for all kinds of gelatins (Wang et al. 2001). A strong interaction between pig gastric mucins and polymer solutions of gelatin and chitosan at pH 5.5 was also described by Silletti et al. (2007). It is also to note that the ion content of the solvent can mask/inhibit the electrostatic interaction between positively charged gelatin and negatively charged mucin. For instance, when PBS buffer was used as solvent, positively charged gelatin showed no interaction with mucin, although positively charged gelatin was expected to reveal an electrostatic attraction with mucin as in the case of purified water. On the other hand, in our recent study (Çelebioğlu 2016) electrostatic interaction was observed between positively charged BLG and negatively charged mucin dissolved in PBS buffer. Low field NMR results suggested that indeed the interaction of proteins with hydrogen ions in water/solvent systems did not mask/block the BLG-mucin interaction (Çelebioğlu et al. 2015, 2016).

Mucin was identified by van Ruth, Roozen, and Cozijnsen (1995) as the key component in saliva to affect flavor release, by binding to and reducing the release of hydrophobic compounds. Saliva/mucin has been shown to influence both the thermodynamic and kinetic components of flavor release from the gel systems by Boland et al. (2004). The largest effect was observed with the most rigid gel, the gelatin gel, where saliva caused large increases in flavor release, due to an increased surface area for diffusion of flavor compounds. Saliva has ability to enhance the water content of the system, thereby increasing the surface area available for the diffusion of flavor compounds. This effect was found for the gelatin gel since it was a rigid gel and the addition of saliva seems to level out the influence of the gel rigidity (Boland et al. 2004).

The reported interaction of mucin with many biologically important entities including biopolymers prompted the preparation of microspheres from admixtures of mucin and gelatin (a widely used pharmaceutical adjuvant). Ofokansi, Adikwu, and Okore (2007), therefore, aimed to prepare microspheres from admixtures of gelatin and mucin and to evaluate the *in vitro* and *in vivo* delivery of ceftriaxone sodium from these microspheres. They observed that microspheres prepared from admixtures of gelatin and S-mucin adsorbed greater amounts of ceftriaxone ( $p = 0.05$ ) in comparison with those prepared from gelatin alone. Thus, they have concluded that in the presence of mucin, the intermolecular network and possibly other characteristics of gelatin were modified. The water sorption behavior in the two media further confirmed a modification of the gelatin microspheres by the mucin (Ofokansi, Adikwu, and Okore 2007). A possible enhancement of the mucoadhesive



**Figure 8.** Turbidimetric measurement of the interactions of gelatin and aminated gelatin with mucin (type III) in (A) purified water, (B) phosphate-buffered saline (PBS) and (C) simulated gastric fluid (SGF). Mucin solution (0.5 mg/ml) and gelatin solution (1 mg/ml) were mixed at volume ratios between 1:3 to 9:1.  $\Delta A$  represents the difference between the experimental and theoretical absorbances of the mixed solution. Each point represents the mean of three experiments. Key: (□) gelatin (IEP = 5.0); (○) gelatin (IEP = 9.0); (△) aminated gelatin. (Wang et al. 2001).

properties of gelatin microspheres by the soluble portion of porcine mucin due to the modified intermolecular network and possibly other characteristics of gelatin in the presence of mucin was suggested in another study by Ofokansi, Okorie, and Adikwu (2009).

### **Mucin interaction with galectin/lectin (carbohydrate binding proteins)/proline-rich-proteins**

Although mucin plays a key role in the function and properties of mucus, it is the synergistic interaction of mucin with smaller proteins, salts, immunoglobulins, and water, which gives mucus its characteristic slimy, viscoelastic property that is responsible for the exceptional lubricating ability of saliva (Feiler et al. 2007). A number of studies have shown that the rheological properties of mucins vary enormously depending on the type (membrane or secreted) and source (human or animal) and the level of purification, and inclusion of other proteins as well as solution conditions (Bansil,

Stanley, and LaMont 1995; Pearson, Allen, and Hutton 2000; Taylor et al. 2005).

An understanding of the biochemistry behind the known interactions between mucins and other proteins, coupled with an appreciation of their pathophysiological significance, can lend insight into the development of novel therapeutic agents. For instance, cancer-associated alteration in the peripheral carbohydrates of colonic mucins can serve as ligands for galactoside-binding protein galectin-3, which is expressed at higher levels in colon cancer than normal colon, binds to colon cancer mucin. Therefore, binding of galectin-3 to mucin may show therapeutic or preventative promise for colon cancer (Byrd and Bresalier 2004). The binding of galectin-3 is carbohydrate-dependent but is also influenced by the N-terminal domain, since phosphorylation of serine residue 6 reversibly inhibits binding of galectin-3 to colon cancer mucin (Cooper, 2002).

Wirth et al. (2002) investigated both binding rate and specificity of the mucus-lectin interaction; the binding of fluorescent-labeled analogs of plant lectins with different carbohydrate specificity to the pig gastric mucin coated microplates was studied. Even though the viscoelastic properties of the gastrointestinal mucus were not mimicked by this assay, the qualitative composition of pig gastric mucin reflects that of the human one. At neutral pH, the lectin-binding capacity of mucin followed the order wheat germ agglutinin (WGA) (sialic acid and N-acetyl-D-glucosamine)  $\gg$  Ulex europaeus isoagglutinin I (alpha-L-fucose)  $\gg$  lentil lectin (alpha mannose) = potato lectin (N-acetyl-D-glucosamine) > peanut lectin (galactosamine) > Dolichos biflorus agglutinin (N-acetyl-galactosamine). This ranking rather reflects the steric accessibility of the mucus proteins than the molar composition of mucins. As sialic acids often operate as chain terminators at the linear or branched glycoproteins, the WGA-binding was highest. The mucin-lectin interaction is characterized by pH-dependence, specificity, and reversibility (Wirth et al. 2002). The highest WGA binding rates were observed at pH 5. At pH 2, the PGM-bound WGA decreased by about 15% compared to pH 5.0 values and at pH 6.0–7.0, PGM-binding of the lectin was in the range of 60–70% compared to pH 5.0 values. Overall, it is expected that the strong and specific interaction between mucin and lectin-decorated formulations will result in anchoring of the drug delivery system at the site of absorption (Gabor et al. 2004).

Moreover, Dam et al. (2007) indicated that the longer polypeptide chain forms of porcine submaxillary gland mucins bind with higher affinities to the lectins, and that the total numbers of free GalNAc residues in porcine submaxillary gland mucins are important for binding to the lectins. They also proposed the internal diffusion model of a lectin jumping from carbohydrate epitope to epitope in a mucin chain is similar to that for a variety of ligands binding to DNA in which binding and sliding occur along the DNA backbone (von Hippel, 2007; Dam et al. 2007).

Argüeso et al. (2009) investigated a new role for the carbohydrate-binding protein galectin-3 in stabilizing mucosal barriers through its interaction with mucins on the apical

glycocalyx. Using the surface of the eye as a model system, they found that galectin-3 colocalized with two distinct membrane-associated mucins, MUC1 and MUC16, on the apical surface of epithelial cells and that both mucins bound to galectin-3 affinity columns in a galactose dependent manner. These results suggest that galectin-3 plays a key role in maintaining mucosal barrier function through carbohydrate-dependent interactions with cell surface mucins. Regarding the mechanism by which the mucin-galectin-3 interaction contributes to the integrity of the mucosal barrier, they proposed that mucins, which are defined by the presence of amino acid tandem repeat domains with multiple O-glycan chains, form strong complexes with multivalent galectin-3 at the epithelial cell surface, as shown in the model in Figure 9.

Furthermore, Zhao et al. (2010) have shown that the transmembrane mucin protein MUC1 is an endogenous ligand of galectin-3 in human colon cancer cells and that the interaction between MUC1 and galectin-3 occurs via binding of galectin-3 to the oncofetal Thomsen-Friedenreich carbohydrate (Gal $\beta$ 1,3GalNAc $\alpha$ -, T or TF) antigen on MUC1. They mentioned that the galectin-3-MUC1 interaction induces MUC1 cell surface polarization and exposure of the cell surface adhesion molecules (Figure 10).

Overall, it is shown that carbohydrate structures present in the highly glycosylated region of mucins make them potential candidates to interact with the galectin family of carbohydrate binding proteins (b-galactoside-specific lectins). In addition, immunological evidence demonstrates that MUC5B interacts with histatin and proline-rich proteins (PRPs) in human salivary fluid (Lontcheva, Oppenheim, and Troxler 1997). The MUC5B-histatin interaction is a direct protein-protein interaction mediated by the MUC5B Cys1, Cys2, and Cys8a (Lontcheva et al. 2000). By contrast, the interaction sites for PRPs remain uncharacterized. Another secretory mucin, MUC7 also interacts directly with PRP2, an acidic PRP via its N-terminal region (Bruno et al. 2005). In order to identify additional MUC7 interacting proteins, Soares et al. (2003) demonstrated that lactoferrin, an iron-binding protein, interacts directly with MUC7 in a glycosylation-independent manner. In human saliva, alpha-amylase (a glycoside hydrolase) also interacts with MUC5B and MUC7 (Lontcheva, Oppenheim, and Troxler 1997; Bruno et al. 2005). The N-terminal region of MUC7 participates in the direct interaction between these two proteins. However, the nature of the  $\alpha$ -amylase-MUC5B interaction, which was suggested to be non-covalent, remains incompletely characterized. Like histatins, statherin, another salivary protein, interacts directly with MUC5B; the MUC5B Cys8a-subdomain forms the binding site for statherin. The involvement of the MUC5B Cys1-, Cys2- and Cys8a-subdomains in directing interactions with statherin and histatins suggests that these proteins undergo a glycosylation-independent interaction (Senapati, Das, and Batra 2010). However, further studies are needed to clarify the involvement of glycosylation or phosphorylation in the binding events of histatins and statherin with MUC5B Cys1-, Cys2- and Cys8a-subdomains since these regions are predicted to have a low

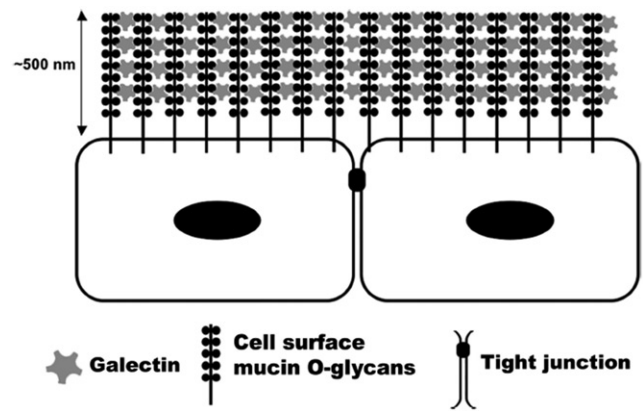


Figure 9. Proposed model of galectin-mucin barrier formation on epithelial surfaces. (Argüeso et al. 2009).

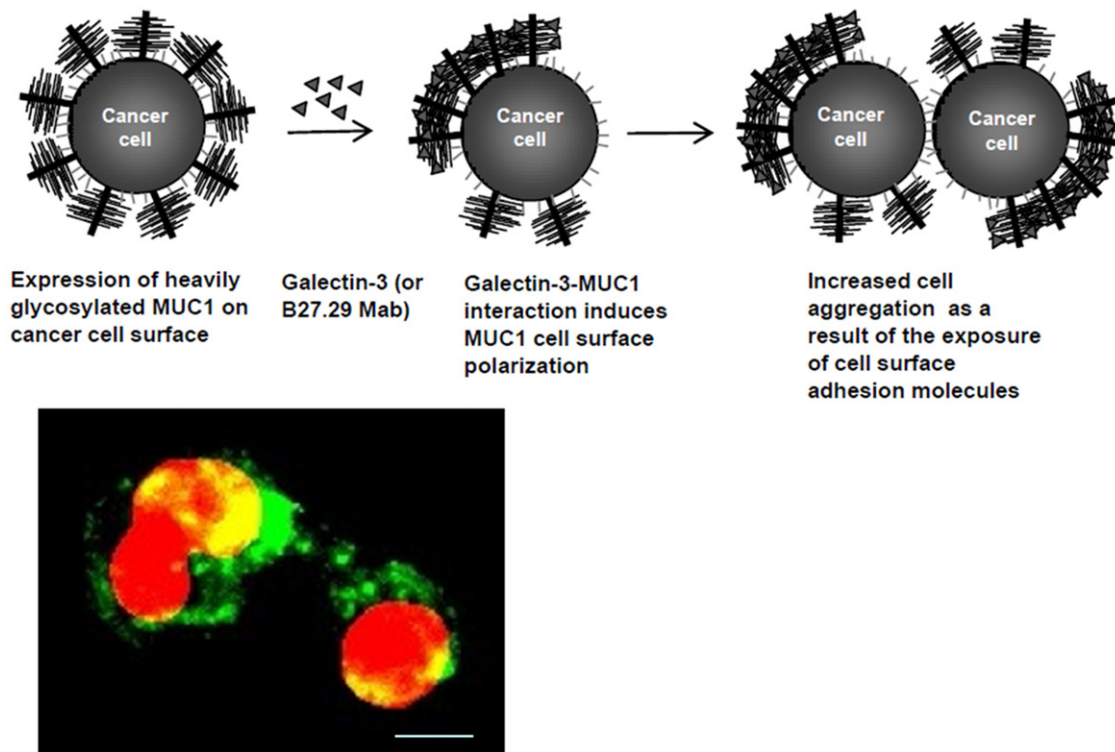
number of potential glycosylation sites in their primary structure (Lontcheva et al. (2000). Interactions between secretory mucins and the aforementioned other salivary proteins seem to be very important in the maintenance of oral physiology (Senapati, Das, and Batra 2010). Experimental evidence indicates that PRP and statherin promote salivary calcium homeostasis. Statherins are also believed to promote the binding of helpful bacteria to the enamel surface. By contrast, the antimicrobial activities of histatins and lactoferrins represent a major component of the non-immune host-defense system in the human oral cavity. Therefore, interactions between salivary mucins and various other salivary proteins might help to enhance their stability and function.

## Concluding remarks

Despite the fact that the physiology of the oral cavity is well studied, little is known about the oral behavior of food proteins and food-protein-stabilized emulsions. Thus, in the last decade there is a growing interest on mucin-protein interaction relating to the food oral processing and digestion. The interactions of salivary mucins and saliva with several food proteins and food protein emulsions, as well as their functional properties related to the food oral processing, were reviewed in this paper. The food proteins of focus were whey proteins (lactoferrin and beta-lactoglobulin) and non-whey proteins (casein, gelatin, galectin/lectin, and proline-rich proteins).

A number of experimental methods have been developed and utilized to study mucin/saliva interactions with food proteins. For instance, sensory analysis with trained panelist, taste sensors, and viscoelastic measurements were used to observe changes in the taste perception after mucin/saliva interaction with food proteins due to the protein aggregation. Tribology tests were developed to measure friction by mimicking the rubbing contacts in oral cavity lubricated by mucin/saliva and/or food protein solution/emulsion. Moreover, several spectroscopies were used to understand in detail the molecular level interaction and the structural changes of proteins. On the other hand, especially for drug delivery purposes, mucoadhesion properties of protein-





**Figure 10.** Representative images of the MUC1 localization in cell aggregates are shown (top). Localization of the cell aggregates-associated MUC1 (green) after treatment of the cells with  $1 \mu\text{g/ml}$  galectin-3 for 48 hr under suspension (red: cell nucleuses) (bottom). Bar =  $10 \mu\text{m}$ . (Zhao et al. 2010).

mucin system via turbidimetric measurements and surface adsorption techniques were commonly used.

Most of the studies suggest that electrostatic attraction is the main mode for the interaction between positively charged proteins with negatively charged moieties of mucin (mainly on glycosylated region of mucin). However, our recent studies related to the BLG-mucin interactions were the first to clearly suggest the importance of hydrophobic as well as the hydrophilic interactions such as hydrogen bonding. Several important subjects, such as hydrophobic surface adsorption, structural and functional changes due to hydrophobic attraction in addition to the hydrophilic ones, were proposed. In addition, the lubrication properties of BLG-saliva systems were determined in terms of strong aggregation between them.

Furthermore, studies of model saliva on the stability of protein emulsions as well as on the effects of food proteins on the adsorption and/or lubrication properties of saliva, suggested the significant differences on the emulsion stability, and/or on the lubrication properties of saliva that were observed when mucin was present in the food-saliva model. This indicates the importance of mucin (rather than saliva) to the mucin/saliva interactions with food proteins. Moreover, the sign and the density of the charge on the surface of the protein emulsion droplets contribute significantly to the behavior of the emulsion when mixed with saliva. Depending on the protein charge, saliva-induced emulsion flocculation is driven by two different main mechanisms, namely, depletion flocculation and electrostatic attraction.

Overall, further studies are required to address in detail the hydrophobic and hydrophilic interactions attraction

between mucin and (food) proteins. Moreover, despite the similar interaction mechanisms of mucin with other proteins and peptides, the conformation of the mucin and the other proteins, after the interaction take place, mostly determine their functional properties.

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