Chapter 5
Protein/Emulsifier Interactions

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5.1 Introduction

Many food emulsions are more complex than a simple colloidal dispersion of liquid droplets in another liquid phase. This is mainly because the dispersed phase is partially solidified or the continuous phase may contain crystalline material, as in ice cream. However, one characteristic that all emulsions have in common is that they are (thermodynamically) unstable. The four main mechanisms that can be identified in the process of breaking down an emulsion are creaming, flocculation, coalescence, and Ostwald ripening. There are two ways in which the process of breakdown of an emulsion can be influenced. First, use of mechanical devices to control the size of the dispersion droplets and second, the addition of stabilizing chemical additives like low molecular weight emulsifiers or polymers to keep it dispersed. The main purpose of the latter is to prevent the emulsion droplets flocculating and from fusing together (coalescence), often achieved by repulsive droplet/droplet interactions. These interparticle interactions are determined mainly by the droplet surface, which is coated with emulsifiers, often surface-active components of biological origin like proteins, mono- and diglycerides, fatty acids, or phospholipids. The forces most commonly observed are electrostatic double layer, van der Waals, hydration, hydrophobic, and steric forces. They are responsible for many emulsion properties including their stability.

The complex mechanisms involved in formation, stabilization, and destabilization of emulsions make fundamental studies on applied systems difficult. One approach has therefore been to clarify the basic physical and chemical properties of emulsions by the study of simpler model systems. The adsorption behavior of single-emulsion components like proteins, fatty acids, surfactants, or phospholipids at liquid/air or liquid/liquid interfaces have given information about surface activity, adsorbed amounts, kinetics, conformation, and surface rheology. The development of experimental techniques has made it possible to extend these studies to multicomponent systems. This has provided further information concerning competitive adsorption, displacement, and complex formation, which can be related to emulsion and foam stability.

For further information concerning the physicochemical factors affecting the emulsion structure as well as characterization of food emulsion stability, the reader

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is referred to the reviews of (Dickinson and Stainsby, 1982; Dickinson, 1996; Wilde, 2000; Bos and van Vliet, 2001a; Benichou et al., 2002; Dickinson, 2003), and for the principles of emulsion formation to the book of Walstra (2003) along with the other chapters in this book. In this chapter we will focus on the molecular interactions between proteins and other surface-active components present at the interface of the emulsion droplets. Understanding the interaction between these emulsifier components is the key to increasing the emulsion stability as well as to be able to tailor the structure of these systems. Various surface-active components like lipids, low molecular weight (LMW) surfactants, and even phospholipids will be regarded as emulsifiers. We will first discuss the stability of the protein in solution, which is an important factor for their behavior in emulsion systems. Although the behavior at liquid/liquid and liquid/air interfaces can be best compared with the situation in an emulsion or foam, we will also discuss some relevant studies concerning the solid/liquid interface as well as the effect of emulsifiers on the solution behavior of proteins.

Surface tension measurements have often been used to study protein–lipid interaction, (cf., Nishikido et al., 1982; Ericsson and Hegg, 1985; Fainerman et al., 1998; Miller et al., 2000a; Vollhardt and Fainerman, 2000). However, it must be born in mind that any impurity with higher surface activity than the studied components will accumulate at the interface giving a lowering of the surface tension (Miller and Lunkenheimer, 1986; Lunkenheimer and Miller, 1987; Lunkenheimer and Czichocki, 1993) and thus affect the interpretation of the data. As an example, the presence of impurities, e.g., fatty acids, bound to β-lactoglobulin did have a profound effect on the interfacial behavior of mixtures with Tween 20, as judged from surface elasticity measurements at the air–aqueous interface (Clark et al., 1995). It was observed that the film containing purified β-lactoglobulin could maintain a more rigid film, at a much higher concentration of Tween 20 as compared to the sample containing impurities. A number of other techniques can also be used to study protein–emulsifier interactions, including surface film balance, ellipsometry, Brewster angle microscopy (BAM), circular dichroism (CD), differential scanning calorimetry (DSC), surface rheology, fluorescence spectroscopy, and neutron reflectivity. It is beyond the scope of this chapter to discuss these techniques in detail, but when necessary a brief explanation will be given.

The link between the molecular interactions between emulsifier components and the properties of food emulsions will be discussed in the last section of this chapter.

5.2 Properties of Proteins and Emulsifiers

5.2.1 Protein Structure and Stability

Relevant aspects of protein aggregation and unfolding are briefly discussed as well as the effects of protein structure (random coil proteins versus globular).

An important consequence of protein–lipid interaction is the effect on stability of the protein in solution as well as on its behavior at interfaces. When discussing the stability of proteins, we may distinguish between the conformational stability of proteins and aggregation/precipitation phenomena due to reduced solubility at pH close to the isoelectric point, at high ionic strength (salting out), and/or caused by specific binding of ions (e.g., the formation of calcium bridges) or lipids. Although the two phenomena usually are connected, aggregation/precipitation can occur without major conformational changes of the protein (Tanford, 1967). The conformational stability of a protein, which of course has no meaning for proteins lacking secondary structure, can be estimated by circular dichroism (cf., Creighton, 1993), compressibility measurement (cf., Gekko and Hasegawa, 1986) and calorimetry (cf., Privalov, 1979; Privalov, 1982; Privalov and Gill, 1988). The stabilization of the protein structure has been extensively reviewed by a number of authors (cf., Privalov, 1979; Privalov, 1982; Privalov and Gill, 1988; Creighton, 1990; Dill, 1990; Ponnuswamy, 1993), and we will only focus on some aspects of significance in emulsion systems.

The native protein structure is a consequence of a delicate balance of forces including electrostatic forces, hydrogen bonding, van der Waals forces, conformational entropy and so-called hydrophobic interactions (cf., Richards, 1977; Pace et al., 1981; Privalov and Gill, 1988; Dill, 1990; Ponnuswamy, 1993). The amino acid sequence of the polypeptide chain (the primary structure) will determine the folding into structural units (the secondary structure) and the association of structural units into domains, tertiary and quaternary structures, gives each protein the unique conformation that is required for its function and activity. Naturally, cross-links, such as disulphide bridges, increase the stability of a protein.

The interior of a globular protein is very densely packed, having a quite constant mean packing density (0.74), a value also found for crystals of small organic molecules (Richards, 1977). Thus, van der Waals forces and hydrogen bonding, which are short range interactions, play an important role for the stability of folded proteins (Privalov and Gill, 1988). As first pointed out by Kauzmann (Kauzmann, 1959), it is clear that the so-called hydrophobic interactions play an important role in stabilizing the protein structure. The nonpolar amino acid residues will provide a strong driving force for folding, leading to an accumulation of hydrophobic residues in the core of the protein molecule. The polar amino acid residues (uncharged and charged) will interact favorably with an aqueous solvent and will consequently be located on the outside of the protein. The nature of hydrophobic interactions in this context is not yet fully understood (cf., Privalov and Gill, 1988; Dill, 1990; Ponnuswamy, 1993), since it still is difficult to analyze them separately from other forces contributing to the stabilization of the protein structure (Privalov and Gill, 1988).

It is important to bear in mind that proteins are only marginally stable at room temperature. This means that the exchange of only one amino acid residue, by for instance genetic engineering, might destabilize or stabilize the protein considerably (Matsumura et al., 1988; McGuire et al., 1995b). This can also be achieved by lipid, surfactant and by denaturing agents like urea. In addition, some proteins have as part of their biological role, specific binding sites for lipids. These binding sites can even be specific for a certain class of lipids. Thus it is important to consider protein–lipid

interactions in relation to the features of each individual protein. As discussed extensively by Norde et al. (Norde, 1986; Haynes and Norde, 1994; Norde, 2000), the delicate balance between forces that stabilize and destabilize the protein might be shifted in the proximity of an interface, leading to unfolding upon adsorption. Also the lipid–aqueous interface of self-assembled structures is also an important type of interface where unfolding of the interacting protein can occur. The loss of entropy upon protein folding is the main force counteracting the stabilization of the protein structure (Dill, 1990). Thus, unfolding upon adsorption is an entropically favored process (Norde, 1986; Haynes and Norde, 1994; Norde, 2000). Furthermore, at an interface the unfolded hydrophobic domains might be oriented in such a way that their exposure to the aqueous environment is minimized. In fact, Norde argues that the entropy gained by the unfolding of the protein upon adsorption can be a significant driving force for adsorption (Norde, 1986; Haynes and Norde, 1994; Norde, 2000). However, they also observed that adsorption of protein on apolar surfaces might lead to an increase in the amount of the protein secondary structure as observed for enzymes like α-chymotrypsin and serine proteinase savinase on Teflon (Maste et al., 1997; Zoungrana et al., 1997; Norde, 2000).

The folding and unfolding of proteins have been shown, under certain conditions, to occur via an intermediate state, the so-called molten globule state (Dolgikh et al., 1981; Ohgushi and Wada, 1983; Dolgikh et al., 1985; Kuwajima, 1989; Ptitsyn et al., 1990; Dickinson and Matsumura, 1994). This state, which is somewhere between the native and completely unfolded state, is characterized by a retained secondary structure, but with a fluctuating tertiary structure. The protein molecule is also more expanded and is exposing more hydrophobic domains. The molten globule state is hard to detect by calorimetric measurements, since the unfolding of the molten globule is accompanied with little or no heat absorption (Kuwajima, 1989). As discussed by Dickinson and Matsumura (Dickinson and Matsumura, 1994), the molten globule state can be achieved in a number of ways, as pH-changes, increase of temperature, the use of denaturation agents, breaking of disulphide bridges and removal of ligands or cofactors bound to the protein. For instance it has been reported that the calcium free form of α-lactalbumin is more hydrophobic (Lindahl and Vogel, 1984). Proteins might also adopt a molten globule state when interacting with an interface. In fact, it was found that α-lactalbumin was more surface active under conditions where it exists in the molten globule state (Engel et al., 2002). This is demonstrated in Fig. 5.1 showing that the adsorption of α-lactalbumin is enhanced as pH is reduced so that the protein structure tends towards that of the molten globule state. It has been proposed that the molten globule state of the protein may be required for the translocation of proteins across biological membranes (Bychokova et al., 1988; van der Goot et al., 1991). The importance of the protein structure in this context was provided by Hanssens and Van Cauwelaert (1978), who studied the penetration of α-lactalbumin in monolayers of DPPC and cardiolipin at physiological pH (pH 7.4) and at pH 4.6 with and without calcium. Indeed, penetration occurred at low pH, when the protein is supposed to be in the molten globule state and was prevented if the protein was adsorbed from a calcium solution (Hanssens and Van Cauwelaert, 1978). The conformation of the protein does not always change significantly when interacting with the lipid monolayer. By recording

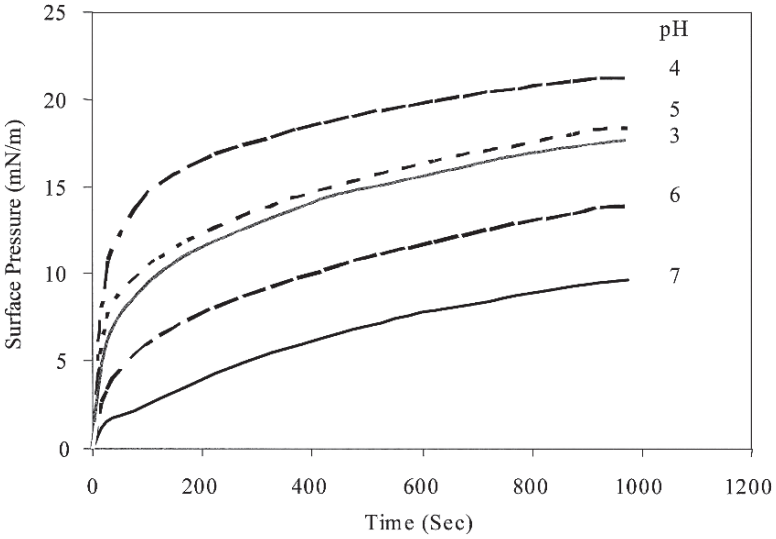

Fig. 5.1 Surface pressure of adsorbing α-lactalbumin as a function of solution pH. The increase in adsorption rates as pH is reduced is initially due to reduction in inter-molecular repulsion as the pH approaches the isoelectric point for α-lactalbumin (pH 4.2). Below this pH, the enhanced adsorption is increasingly due to molecular unfolding as the protein structure tends towards the molten globule state

CD-spectra for β-lactoglobulin, α-lactalbumin or BSA bound to mixed monolayers of POPC and POPG and transferred to a quartz plate, Cornell et al. showed (Cornell and Patterson, 1989; Cornell et al., 1990) that the protein bound to the lipid monolayer was similar to the one recorded in solution.

Protein properties as conformation, charge distribution, association and activity are also strongly influenced by environmental condition, e.g., pH, ionic strength, type of ion and temperature. In this context it is important to point out the effect of type, valence and ionic strength of added electrolyte. As discussed by Ninham et al. this can have profound effect on interactions involving proteins and other polyelectrolytes, in particular under physiologically relevant conditions (Boström et al., 2001; Boström et al., 2002; Ninham, 2002). They argue that the present theory is not adequate to distinguish between van der Waals interactions and electrostatic interactions under these conditions.

5.2.2 Emulsifiers and Their Phase Behavior

Different types of emulsifiers are defined I) aqueous soluble, surfactant type and II) lipids with low aqueous solubility. The self-assembled structures formed by the different types of surfactants are discussed.

Lipids can be divided into two major groups: polar and nonpolar lipids. The nonpolar lipids, primarily the triglycerides, have small polar groups, and hence show only limited interaction with aqueous systems. The polar lipids, however, with large charged or uncharged polar groups, giving these lipids amphiphilic nature, associate in aqueous systems. The common feature for the self-assembly of the polar lipids in aqueous environment is the formation of a polar interface, which separate the hydrocarbon and water regions. The hydrocarbon chains can exist either in a fluid state, as in liquid crystalline phases, or in a solid state, as in the lipid gel phases (Larsson, 1994). Generally, the melting of the chains in an aqueous environment occurs at a much lower temperature compared to the melting of the pure lipid.

It is convenient to distinguish between surfactants/polar lipids according to their water solubility:

1. Polar lipids and synthetic analogues, i.e., surfactants, that are water soluble in monomeric and micellar form,
2. Polar lipids with very low water solubility, but with the ability to swell into liquid crystalline phases.

The water-soluble polar lipids (e.g., ionized fatty acids, bile salts, and synthetic surfactants, charged or uncharged) have monomeric solubility in the millimolar range and form micelles at higher concentrations. The critical micelle concentration (cmc) is considered to be a narrow concentration range, within which aggregates start to form by a strong cooperative process (Lindman and Wennerström, 1980). The driving force for micelle formation is the hydrophobic interaction (cf., Tanford, 1980). The cmc for single-chain amphiphiles decreases with increasing chain length; and for ionic amphiphiles cmc also depends on the ionic strength, as addition of salt reduces the electrostatic repulsion between the charged head groups. Increased temperature has, however, only a moderate influence on cmc, once the temperature has exceeded the critical temperature, where the monomer solubility is equal to the cmc (Krafft temperature).

A common feature of the two classes of polar lipids is the tendency to form lyotropic liquid crystalline phases. A summary of some of the different liquid crystalline phases that can occur is given in Fig. 5.2. With decreasing water content, the phase behavior of polar lipids often follows the sequence: hexagonal phase (H_I) $\rightarrow$ lamellar phase (L_α) for water soluble lipids and lamellar phase (L_α) $\rightarrow$ reversed hexagonal phase (H_{II}) for lipids with low water solubility. At low water content an inverse micellar structure, the L_2 phase, is formed, in which the hydrocarbon chains form the continuous medium and the aqueous medium is present within the micelles. Cubic liquid crystalline phases (Q) often occur in between these phases. Phase transitions can also occur with changes in temperature; with increasing temperature the sequence of thermal transitions is usually the same as with decreased water content. The formation of a particular phase can in many cases be understood by looking at the geometric packing properties of the amphiphilic molecule in the particular environment (Israelachvili et al., 1976; Mitchell and Ninham, 1981), that is the cross-section area of the polar head group in relation to that of the acyl chain. This property can be expressed by the so called packing parameter (*v/al*), which is defined

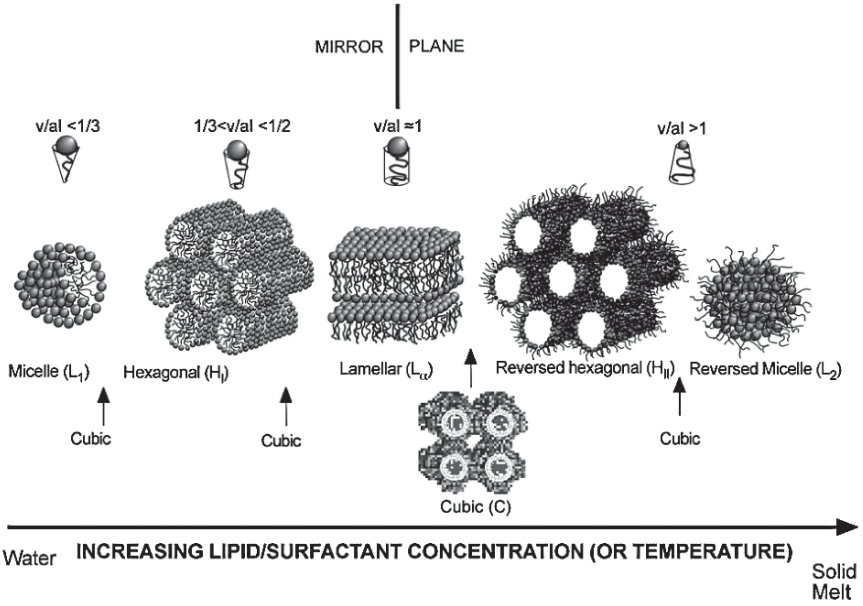

Fig. 5.2 Commonly formed association structures by polar lipids. Phase transitions can be induced by changes in water content, temperature or by interaction with other solution components, like proteins. The lamellar liquid crystalline phase (L_α) can be regarded as the mirror plane, where the aggregates are of the "oil-in-water" type on the water rich side and of "water-in-oil" type on the water poor side (Fontell, 1992). On both the water rich and water poor sides of the L_α there are two possible locations for cubic phases. Other "intermediate phases" may also occur. The formation of a particular phase can in many cases be understood by looking at the geometric packing properties of the amphiphilic molecule in the particular environment (Israelachvili et al. 1976; Mitchell and Ninham, 1981). This property can be expressed by the so called packing parameter (*v/al*), which is defined as the ratio between the volume of the hydrophobic chain (*v*) and the product of the head group area (*a*) and the chain length (*l*)

as the ratio between the volume of the hydrophobic chain (*v*) and the product of the head group area (*a*) and the chain length (*l*). The packing parameter for a particular environment will determine the curvature of the interface and thus the particular phase. Generally speaking (see Fig. 5.2), a value of the packing parameter lower than unity (cone shaped amphiphile) facilitates the formation of structures where the polar interface is curved towards the hydrocarbon phase, i.e., structures of "oil-in-water" type (L_1, H_I). On the other hand a value larger than one (reversed cone shaped amphiphile) will give the reverse curvature and favor "water-in-oil" structures like H_{II} and L_2. When the packing parameter is changed by for instance the change of ionic strength, temperature or the addition of other molecules like proteins, phase transitions will ultimately arise. Increased temperature, e.g., will increase chain mobility and thereby increase the volume of the lipophilic part of the molecules, explaining the often seen thermally induced transition $L_\alpha \rightarrow H_{II}$. Decreased hydration

will decrease the head group repulsion, resulting in a decreased interface area and thus in an increase of the packing parameter.

In nature and in many technical applications the lipid aggregates consist of a mixture of different lipids, which either exist in a homogenous mixture or separate into domains. As discussed in the review by Raudino (Raudino, 1995), the lateral distribution in these mixed aggregates is influenced by a number of factors like ionic strength, presence of polymers/proteins as well as the composition of the lipids and it is thus hard to give any general rules to predict when phase separation will occur.

Luzzati and coworkers determined the main features of the most commonly found mesophases in the early 1960s by X-ray diffraction (reviewed by Luzzati in 1968). Results from spectroscopy studies have increased the understanding of the dynamic nature of these phases. The lamellar phase (L_α) consists of stacked infinite lipid bilayers separated by water layers, while the hexagonal phases consists of infinite cylinders, having either a hydrocarbon core (H_I) or a water core (H_{II}). As shown in Fig. 5.2, the cubic phases (C) can exist in several locations in the phase diagram and have been shown to exist in a number of lipid systems (Fontell, 1990; Templer, 1998). They are isotropic and highly viscoelastic. Different structures of the cubic phases, depending on the particular lipid system, have been suggested (Luzzati et al., 1968; Larsson, 1989; Lindblom and Rilfors, 1989; Fontell, 1992; Hyde et al., 1997; Templer, 1998):

1. *Bicontinuous cubic phase* that consists of curved nonintersecting lipid bilayers, forming two unconnected continuous systems of water channels (cf., Lindblom et al., 1979; Larsson, 1989; Templer, 1998). If an interface is placed in the gap between the methyl end groups of the lipid in the bicontinuous bilayer type of cubic phase, it will form a plane that can be described as a minimal surface (Andersson et al., 1988; Larsson, 1989). This type of cubic phase, C_{bic}, has been observed in aqueous dispersions of polar lipids with low aqueous solubility like monoglycerides, phospholipids and glyceroglucolipids (Larsson, 1989; Fontell, 1990; Larsson, 1994) as well as for water soluble surfactants like ethoxylated fatty alcohols (Wallin et al., 1993).
2. *The discerete type of cubic phase* was first suggested by Luzzati et al. (1968). The occurrence of micellar cubic phase, C_{mic}, where disjointed reversed micelles embedded in a three-dimensional hydrocarbon matrix are organized in a cubic symmetry, space group Fd3m, has been reported by Luzzati and coworkers (Luzzati et al., 1992). The formation of this type of C_{mic} phases has been reported for aqueous systems containing monoolein and oleic acid (Mariani et al., 1988; Mariani et al., 1990; Luzzati et al., 1992; Borné et al., 2001), for aqueous mixtures of sodium oleate and oleic acid (Seddon et al., 1990), and consequently also during lipase catalyzed lipolysis of monoolein in aqueous dispersions under neutral /alkaline conditions (Borné et al., 2002a; Caboi et al., 2002).

Today, cubic lipid-aqueous phases are recognized as important in biological systems (Mariani et al., 1988; Larsson, 1989; Lindblom and Rilfors, 1989; Seddon, 1990; Larsson, 1994; Landh, 1995; De Kruijff, 1997; Hyde et al., 1997; Luzzati,

1997; Templer, 1998; Larsson, 2000; Larsson et al., 2002). Some of these reports suggest that cubic lipid-aqueous phases can occur during the fusion of biological membranes. There are a vast amount of studies of membrane fusion (cf., the comprehensive reviews by Kinnunen and Holopainen, 2000), which is impossible to cover here. The liquid-crystalline lipid aqueous phases can exist in excess of aqueous solution. One example of such lipid dispersions is vesicles or uni- or multilamellar vesicles,[1] which is formed from lamellar (L_α), phases. The stability, size and shape of vesicles can vary, depending on the composition of lipids and aqueous phase (for reviews see for instance Helfrich 1989; Lasic 1993; Komura 1996; Lasic et al. 2001). In analogy with liposomes, dispersions of a cubic lipid-aqueous phases, Cubosome®[2] particles, which were first discovered by Larsson et al. (Larsson, 1989; Landh, 1994; Larsson, 2000) are also formed in excess of water. The stability of Cubosome® particles, formed in monoolein -H_2O-based systems, and the corresponding dispersed H_{II} phase (Hexosome® particles) in the monoolein-triolein-H_2O system were found to increase in the presence of an amphiphilic block-copolymer (polyoxamer) (Landh, 1994; Gustafsson et al., 1996; Gustafsson et al., 1997). Since the early work of Larsson et al. several studies on different types of dispersed liquid-crystalline nanoparticles have been presented with focus on systems for drug delivery as well as delivery of functionality to foods (Barauskas et al., 2005a; Barauskas et al., 2005b; Esposito et al., 2005; Spicer, 2005a; Spicer, 2005b; Almgren and Rangelov, 2006; Angelov et al., 2006; Barauskas et al., 2006a; Barauskas et al., 2006b; Boyd et al., 2006; Johnsson et al., 2006; Sagalowicz et al., 2006a; Sagalowicz et al., 2006b; Tamayo-Esquivel et al., 2006; Vandoolaeghe et al., 2006; Worle et al., 2006; Yaghmur et al., 2006).

5.3 Protein/Emulsifier Interaction in Solution

5.3.1 Aqueous Soluble–Surfactant Type of Emulsifiers

The monomer concentration (defined by cmc) is an important parameter for the interaction between the emulsifier and the protein.

Ionic surfactants interact with most proteins. High surfactant concentrations will generally lead to unfolding of the protein structure. The interactions between nonionic surfactants and proteins are weaker and seldom affect the structure of proteins. Several reviews concerning the interaction between water-soluble polar lipids and protein are focused on the interaction between ionic surfactants, e.g., sodium dodecylsulphate (SDS), and globular proteins at low and intermediate temperatures

[1] The term liposomes is according to IUPAC recommendation synonymous to lipid vesicles, but is sometimes used for multilamellar vesicles.

[2] Cubosome® and Hexosome® are registered trade names for Camurus AB, Sweden.

(Steinhardt and Reynolds, 1969; Lapanje, 1978; Makino, 1979; Jones and Brass, 1991; Ananthapadmanabhan, 1993; Dickinson, 1993; Bos et al., 1997; Dickinson, 1999). Since vast amount of the surfactant-protein work is devoted to SDS, we will use this system as an example and at the end of this section we will discuss some exceptions.

We can distinguish between two types of binding of surfactants to proteins:

1. A high affinity type of binding that occurs at low lipid concentration (Jones and Brass, 1991)
2. Nonspecific cooperative interaction taking place at higher concentrations (Jones and Brass, 1991; Ananthapadmanabhan, 1993).

An example of a binding isotherm, where the two types of binding occur, is given in Fig. 5.3. In this isotherm, for the binding of sodium dodecylsulphate (SDS) to

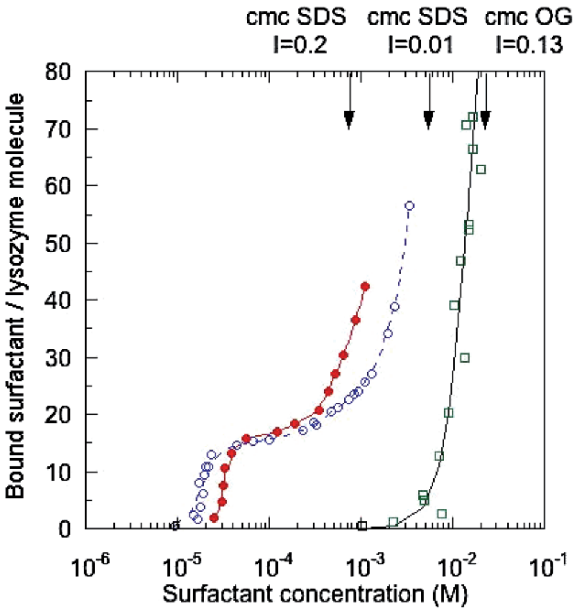

Fig. 5.3 Binding isotherms for binding of surfactants to lysozyme in aqueous solution at 25 °C. The isotherms (❍, ●) for sodium dodecylsulphate (SDS) have regions of both high affinity non-cooperative binding, at low surfactant concentration, and cooperative binding at high concentration. The influence of ionic strength on the binding isotherm is shown: ●, ionic strength (I) 0.0119 M and ❍, ionic strength 0.2119 M at pH 3.2. For comparison, an example of a binding isotherm where only nonspecific cooperative binding occurs, is also inserted. This isotherm, describing the binding of the nonionic n-octyl-β-glucoside (OG) to lysozyme (❑) was measured at pH 6.4, ionic strength 0.132 M. The protein concentration was 0.13% w/v. The arrows indicate cmc for the different surfactants and ionic strengths. The data is adapted from Jones (Jones and Brass, 1991) and the experimental details are given in references (Jones et al. 1984) and (Jones and Brass, 1991) for SDS and OG, respectively

lysozyme, the region of high affinity non-cooperative binding, at low surfactant concentration, is well separated from the cooperative binding observed at higher concentration. For comparison an example of a binding isotherm for the binding of a nonionic surfactant, n-octyl-β-glucoside, to the same protein, is also inserted. In this case only nonspecific cooperative binding occurs.

5.3.1.1 Specific Binding of Proteins and Emulsifiers

The specific binding is mediated by ionic and hydrophobic interactions and usually occurs below the cmc of the surfactant (Yonath et al., 1977a; Yonath et al., 1977b; Jones and Manley, 1979; Jones and Manley, 1980; Jones and Brass, 1991). There are many examples of proteins that possess binding activity, including bovine serum albumin and β-lactoglobulin. Investigation of the binding properties of these proteins has been generally confined to studies in bulk solution. For example, the presence of a fluorescent tryptophan residue in the hydrophobic cleft of β-lactoglobulin (Papiz et al., 1986) has facilitated the study of emulsifier binding by fluorescence titration. Subsequent analysis of binding by conventional methods such as that of Scatchard (Scatchard, 1949) allows determination of the dissociation constant (K_d) of the complex formed. Typical examples of K_d'*s* for β-lactoglobulin are shown in Table 5.1. The effect of complex formation can usually be detected by shifts in the surface-tension (γ) curve (Dickinsson and Woskett, 1989). An example of this is shown for Tween 20 and β-lactoglobulin in Fig. 5.4 (Coke et al., 1990). Surface-tension/concentration (γ-c) curves for Tween 20 alone and in the presence of a fixed concentration of β-lactoglobulin (0.2 mg/ml; 10.9 μM) are shown.

The general features described earlier are evident with a comparatively low concentration of protein causing a significant reduction in γ. In the absence of protein, γ reduces gradually with increasing Tween 20 concentration. The gradient of the reduction in surface tension reduces at higher Tween 20 concentrations (>30 μM) but

Table 5.1 Typical dissociation constants of emulsifier/β-lactoglobulin complexes

Emulsifier	Dissociation constant	References
Tween 20	4.6 μM	Wilde and Clark, 1993
L-α lysophosphatidyl-choline, palmitoyl	166 μM	Sarker et al., 1995
Sucrose monolaurate	11.6 μM	Clark et al., 1992
Sucrose monostearate	1.02 μM	Clark et al., 1992
Sucrose monooleate	24.8 μM	Clark et al., 1992
Sodium stearoyl lactylate, pH 7.0	0.26 μM	Clark, unpublished
Sodium stearoyl lactylate, pH 5.0	0.30 μM	Clark, unpublished
Lauric acid	0.7 μM	Frapin et al., 1993
Palmitic acid	0.1 μM	Frapin et al., 1993

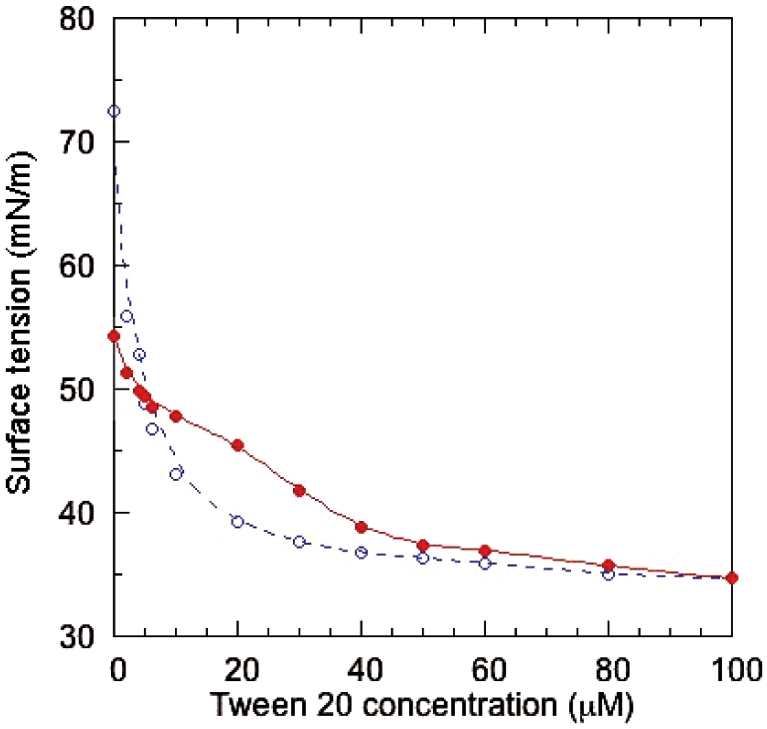

Fig. 5.4 Surface tension isotherm for Tween 20 in the absence (●) and presence (❑) of 0.2 mg/mL β-1actoglobulin. The data were recorded after 20 min adsorption and are therefore not at equilibrium

doesn't become completely flat due to failure to attain equilibrium γ, possibly due to the presence of a mixture of surface-active species in the Tween 20 sample. In contrast, the curve in the presence of protein maintains a relatively steady surface-tension value of about 50 mN/m up to Tween 20 concentrations of 25 μM due to the surface tension reduction caused by adsorption of the protein. This means that the curve for the sample containing protein crosses that of Tween 20 alone. This is strong evidence for complex formation between the two components, since the curves cross due to a reduction in the concentration of free emulsifier in solution as a fraction of the emulsifier interacts with the protein to form the complex.

Thus, great care must be taken when considering the surface properties of compounds in solutions containing mixtures of interacting components. In the simplest case of a single binding site, the two-component system becomes a three-component system comprising free emulsifier, free protein, and emulsifier/protein complex. The relative proportions of the components present can be calculated in the following manner (Clark et al., 1992). In the simplest case, the interaction of an emulsifier (E) with a protein (P) can be described by the expression

$$P + E \leftrightarrow PE \tag{5.1}$$

where PE is the emulsifier/protein complex. Thus the dissociation constant *(K_d)* for the complex can be expressed as

$$K_d = \frac{[P][P]}{[PE]} \tag{5.2}$$

where the square brackets indicate molar concentrations of the different species. It is also the case that

$$[\mathrm{P}] = [\mathrm{P_{tot}}] - [\mathrm{PE}] \tag{5.3}$$

$$[\mathrm{E}] = [\mathrm{E_{tot}}] - [\mathrm{PE}] \tag{5.4}$$

where $[P_{tot}]$ and $[E_{tot}]$ are the total protein and emulsifier in the system. Substituting Eqs. (5.3) and (5.4) in Eq. (5.2) gives

$$[\mathrm{PE}] - ([\mathrm{E_{tot}}] + [\mathrm{P_{tot}}] + K_d)[\mathrm{PE}] + [\mathrm{P_{tot}}][\mathrm{E_{tot}}] = 0 \tag{5.5}$$

which can be solved for [PE] and can be used to calculate the relative concentrations of the three components. In addition, the binding data, which may comprise a change in a parameter (e.g., intrinsic fluorescence) caused by formation of the complex may be fitted using this equation, provided there is a single active binding site and the titration is carried out to saturation. Alternatively, it is possible to determine the dissociation constant and number of binding sites from the Scatchard equation (Scatchard, 1949)

$$\frac{v}{[E]} = \frac{n - v}{K_d} \tag{5.6}$$

where *v* is the fraction of protein with occupied sites (i.e., $[PE]/[P_{tot}]$). If the Scatchard plot of *v* against *v/[E]* gives a straight line, it indicates the presence of only one class of binding sites. The gradient of this line is $1/K_d$, and the intercept on the x axis gives the number of binding sites, *n*. If the Scatchard plot does not give a straight line, then the shape of the curve obtained can be used to identify if the observed binding is positively or negatively cooperative or the presence of multiple independent sites. In the former case the Hill equation can be used to determine the K_d and a cooperativity coefficient (Hill, 1910).

5.3.1.2 Nonspecific Interaction

The nonspecific interaction often occurs close to the cmc as it is associated with the aggregation of the surfactant and usually leads to a destabilization of the native conformation. The cmc of the surfactant is thus an important parameter and conditions that affect cmc will generally affect the binding, (cf., Ananthapadmanabhan, 1993; Waninge et al., 1998). The saturation of all the binding sites generally corresponds to 1–2 g of surfactant per gram of protein (Reynolds and Tanford, 1970; Jones and Brass, 1991; Ananthapadmanabhan, 1993).

The extent of interaction and unfolding depend mainly on the nature of the surfactant hydrophilic group, surfactant chain length, ionic strength, pH, temperature and organic additives as well as on the protein itself (Ananthapadmanabhan, 1993). Organic additives include the presence of impurities in proteins as well as in the lipids. For instance, it has been demonstrated by Lunkenheimer and coworkers that commercial SDS samples usually contains a substantial amount of dodecanol, which actually is more surface active than SDS by itself (Miller and Lunkenheimer, 1986; Lunkenheimer and Miller, 1987; Lunkenheimer and Czichocki, 1993). Similarly, it has been shown by Clark et al. that β-lactoglobulin contains bound fatty acids, which may alter the binding of other surface active compounds (Clark et al., 1995). Clearly, the presence of amphiphilic impurities may give anomalous effects on the binding of other surfactants.

The effect of surfactant protein interaction on the structural stability of proteins depends strongly on the mode of interaction. In fact as shown in Fig. 5.5, the same surfactant can act as both stabilizing and destabilizing depending on surfactant concentration as well as other solution conditions. At low surfactant-to-protein ratios, high affinity interaction between certain proteins and surfactants occur. This interaction stabilizes the protein structure against thermally induced unfolding, thus the thermally induced transition is shifted towards higher temperature as illustrated in Fig. 5.5 and previously reported by Hegg (Hegg, 1980) for SDS and β-lactoglobulin, Similar findings has also been reported for other protein–surfactant complexes such as between fatty acids or SDS and bovine serum albumin (Gumpen et al., 1979) as well as between palmitic acid and β-lactoglobulin (Puyol et al., 1994). As discussed above increasing the free surfactant concentration to the cmc give rise to nonspecific cooperative binding, which in turn can lead to unfolding of the protein as illustrated in Fig. 5.5 (Waninge et al., 1998). This is in agreement with earlier reports, where total surfactant ratio above 10 moles of SDS per mole of serum albumin or 1 mole of SDS per mole of β-lactoglobulin monomer were observed to cause unfolding of the protein (Gumpen et al., 1979; Hegg, 1980).

Anionic

Surfactants like alkylsulphates or alkylethersulphates interacting with proteins with opposite net charge, e.g., lysozyme or gelatine, might cause precipitation of the protein–surfactant complex due to neutralization of the net charge (Jones and Manley,

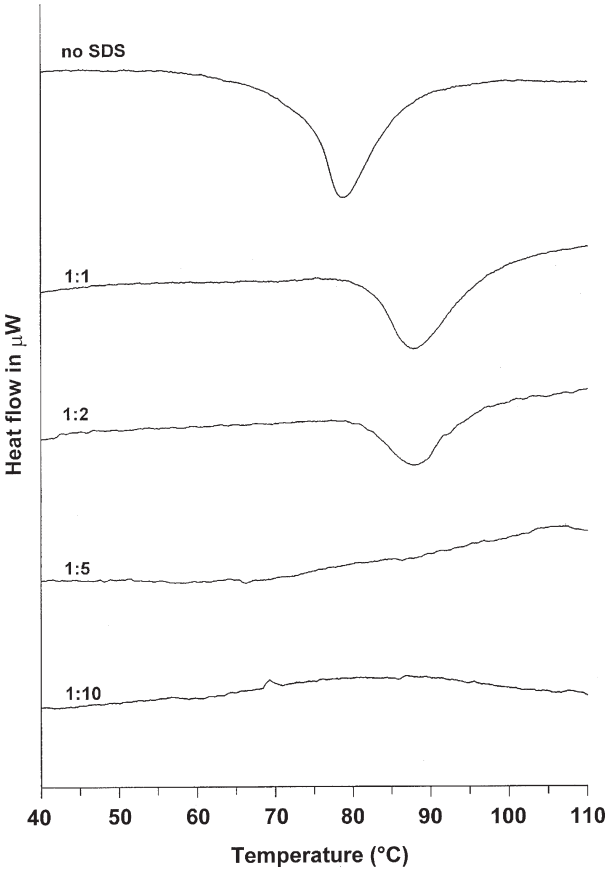

Fig. 5.5 The thermograms from top to bottom shows the thermally induced unfolding of β-lactoglobulin (1.4 mM in 60-mM NaCl, pH 6) when increasing the protein/SDS molar ratio. The cmc of SDS is 0.47 mM at 25 °C and ≈1 mM at 90 °C, when taking into account the ionic strength of the protein solution. Assuming that 1 SDS molecule is bound per β-lactoglobulin monomer, 3-mM SDS has to be added to reach the cmc of the surfactant at 90 °C. The data are adapted from (Waninge et al. 1998), where also the experimental details are given

1979; Fukushima et al., 1981; Fukushima et al., 1982; Chen and Dickinson, 1995a; Chen and Dickinson, 1995b; Morén and Khan, 1995; Stenstam et al., 2001). Although the protein is precipitated, usually only small changes in the secondary structure occur. At an increased surfactant concentration the complex is dissolved and the protein starts to be unfolded. Generally, denaturation of proteins by long-chain alkyl sulphates such as SDS results in a structure with large fractions of the polypeptide chain in an α-helical conformation (Jirgensons, 1976; Mattice et al., 1976; Tanford, 1980). As a simple rule, proteins with a low content of α-helix in

their native form, such as concanavalin A, β-lactoglobulin and ovalbumin, will increase in α-helix content upon interacting with SDS. The reverse is observed for proteins with a high α-helix content in their native form, e.g., myoglobin and serum albumin (Mattice et al., 1976). The structure resulting from the interaction is thought to consist of helical segments with flexible joints, and with most of the hydrophobic side-chains exposed to the surfactant. The successive binding of SDS opens up the molecules, due to the increased electrostatic repulsion, and unveils new hydrophobic domains, which can bind additional surfactants. This association stabilizes α-helical folding at the expense of nonrepetitive structure. The free energy gained by this process in most cases by far exceeds the unfavorable free energy change of disrupting the native conformation (Tanford, 1980). Light scattering studies confirm the expansion of the hydrodynamic radius of the protein upon interaction with SDS (Tanner et al., 1982). Several models of the structure of complexes between SDS and proteins at high surfactant concentration, like the correlated necklace, rod-like structure and flexible helix, have been considered, (cf., Guo and Chen, 1990; Ananthapadmanabhan, 1993). However, small-angle neutron scattering data strongly indicates a structure resembling a necklace (Guo and Chen, 1990; Guo et al., 1990), where the polypeptide chain with high flexibility is decorated with SDS micelles (Mattice et al., 1976; Guo and Chen, 1990) as shown in Fig. 5.6. This interaction is reported to take place via the monomeric form of the surfactant (Mattice et al., 1976; Ananthapadmanabhan, 1993).

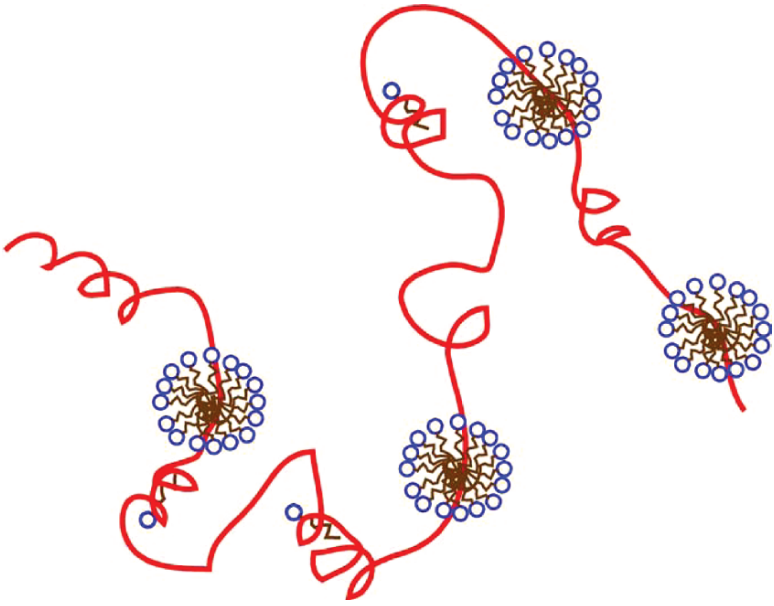

Fig. 5.6 Schematic representation of the so-called necklace model for the interaction between SDS and proteins. The solid line represents the unfolded polypeptide chain, which still contains secondary structure. Micelle-like clusters are cooperatively formed on the polypeptide chain

It should also be born in mind that not all proteins are fully unfolded by SDS. For instance it has been shown that the activities of glucose oxidase, papain, pepsin and bacterial catalase were not affected by high concentration of SDS, correlated to the low binding of SDS (Nelson, 1971; Jones et al., 1982).

Within the type of surfactant the binding is dependent on the nature of the polar head group, e.g., for anionic surfactant the interaction decreases in the order alkyl sulphates > alkyl sulphonates > alkyl benzene sulphonates > carboxylates ≈ alcohols (Reynolds et al., 1968; Rendall, 1976).

Nonionic

The interaction between nonionic surfactants and proteins are generally weak (Reynolds et al., 1968; Green, 1971; Makino et al., 1973; Sukow et al., 1980; Cordoba et al., 1988; Bos et al., 1997). They are therefore often used to solubilize/stabilize proteins in biochemical preparations, e.g. (Ahlers et al., 1990). For instance, each β-lactoglobulin monomer binds only one Tween 20 (Wilde and Clark, 1993), or one sucrose ester (Clark et al., 1992) or one Triton X-100 (Green, 1971). Generally, minor changes of the structure upon interaction are observed (Makino et al., 1973; Cordoba et al., 1988). An unordered, flexible protein, β-casein, was found to bind less than one sucrose ester per protein molecule, possible due to incorporation of the surfactant in β-casein micelles (Clark et al., 1992). The specific ionic interaction present for ionic surfactants in addition to the hydrophobic interaction that leads to more severe effects on the protein structure, is absent for the nonionic surfactants (Fig. 5.3). Another reason for the weaker interaction between proteins and nonionic surfactants has been assigned to the lower cmc, which gives fewer monomers in the solution that can bind to the protein (Makino et al., 1973). The cmc is increased when the cha≈in length is decreased, which may change this situation; the binding of octyl glucoside to various proteins was found to occur in a cooperative manner at surfactant/protein molar ratio of hundred and more, without any evidence of protein denaturation (Cordoba et al., 1988).

Also the nature of the nonionic polar head groups will affect the interaction. For a series of Triton X surfactants increasing the hydrophilic oxyethylene chain length was found to decrease the strength of interaction with BSA, due to steric hindrance as well as relatively lower hydrophobicity (Sukow et al., 1980). The calorimetric data indicated that some conformational changes of BSA occurred during the saturation of the low affinity, non-cooperative binding sites (Sukow et al., 1980).

Some studies have also been carried out with the zwitterionic surfactant lysophosphatidylcholine (LPC), which was found to bind cooperatively to puroindoline, a lipid binding protein isolated from wheat flour, at a molar ration of 5 to 1 (Wilde et al., 1993), with an affinity that was dependent on the chain length of the LPC molecule (Husband et al., 1995). One LPC molecule was also found to bind with less affinity to β-lactoglobulin than Tween 20 (Sarker et al., 1995). The binding of Tween 20, as opposed to LPC, had a much more disruptive effect on the interfacial film of the protein, attributed to the bulkier head group of Tween 20. This implies that a

nonionic surfactant can also disrupt the structure of a protein, provided that the binding is strong enough and the hydrophilic head group large enough to sterically induce conformational changes.

Cationic

Cationic surfactants generally seem to exhibit an intermediate action on water-soluble proteins. Reports in the literature indicate a cooperative interaction with proteins, but with less affinity and thus with less perturbation of the folded state, compared to the effect of the anionic ones (Tanford and Epstein, 1954; Kaneshina et al., 1973; Nozaki et al., 1974; Ericsson et al., 1987a; Ericsson et al., 1987b; Waninge et al., 1998). If the binding is governed both by electrostatic and hydrophobic interactions, anionic and cationic surfactants will obviously occupy different sites. Nozaki et al. has suggested that the lower affinity of many proteins for cationic compared to anionic surfactants, can be explained by the fact that the cationic arginine and lysine side chains contributes with more CH_2 groups than anionic aspartate and glutamate side chains (Nozaki et al., 1974). This implies that the combined electrostatic and hydrophobic interactions and possibly accessibility of the charged groups will be more favorable for anionic surfactants. As a consequence, the cooperative binding step will start at a higher concentration for cationic relative to anionic surfactants (Ananthapadmanabhan, 1993).

5.3.1.3 Effect of Solution Conditions

Increased ionic strength can affect the interaction between protein and ionic surfactants by reducing the electrostatic attraction between surfactants and amino acid residues with opposite net charges. Generally, the high affinity non-cooperative binding is strongly influenced by the electrostatic interaction between surfactant and protein. Thus this part of the binding isotherm will be shifted towards higher surfactant concentration upon addition of salt, as observed for lysozyme and SDS (Fig. 5.3) (Jones et al., 1984; Jones and Brass, 1991). Increasing the ionic strength, will on the other hand, favor the cooperative binding by screening the repulsion between the charged surfactant head groups. This part of the surfactant binding isotherm will therefore be shifted towards lower surfactant concentrations, parallel to the decrease of surfactant cmc. Here it is important to point out that the presence of highly charged proteins will affect the formation of micelles in the same way as a polyelectrolyte as well as the effect of temperature. This has been amply demonstrated by Waninge et al. who studied thermally induced unfolding of β-lactoglobulin at a concentration of 1.4 mM in 60-mM NaCl, pH 6, at various molar ratios of SDS and their main findings are illustrated by the thermograms, obtained by differential scanning calorimetry (DSC), in Fig. 5.5 (Waninge et al., 1998). From this figure we note that the peak corresponding to the thermal unfolding disappears when the protein/SDS molar ratio increases above 1:2. This corresponds to a SDS concentration of about

3 mM. The cmc for SDS is about 8.1–8.2 mM in water (Williams et al., 1955; Flockhart, 1961). However, the cmc for ionic surfactants decreases with ionic strength and increases with temperature (Williams et al., 1955; Flockhart, 1961; Evans et al., 1984a; Evans et al., 1984b). Taking these effects into account, the presence of β-lactoglobulin (which has a net charge of −5 at pH 7) at a concentration of 1.4 mM in 60-mM NaCl, the cmc of SDS is expected to be 0.47 mM at 25 °C and ≈1 mM at 90°C. When taking into account the specific binding of one SDS molecule per β-lactoglobulin monomer, 3-mM SDS has to be added to reach the cmc of the SDS at 90°C. Thus any affect of nonspecific cooperative interaction between the surfactant and the protein is expected to take place at this SDS concentration. In Fig. 5.5 we observe an apparent loss of protein structure. The unfolding of the protein structure at low temperature, which is observed in the presence of most anionic surfactants such as SDS at high concentration, is expected to be maintained at increased temperature. However, since cmc generally increases with temperature, we might arrive at the situation where the cooperative binding ceases to exist at the high temperature, maybe even below the temperature at which thermally induced unfolding takes place. Interestingly, Waninge et al. observed that the conformational changes invoked by the nonspecific cooperative binding of SDS at 25 °C could be reversed by extensive dialysis (Waninge et al., 1998).

Although cationic surfactants seem to cause less unfolding of globular proteins at low temperature than anionic, some reports indicate that they can destabilize globular proteins at increased temperature (Ericsson et al., 1983a; Ericsson et al., 1987a). However, these reports also indicate that the unfolding process at the same time becomes considerably more reversible. The heat denaturation of ovalbumin, which in practice is completely irreversible, was found to be completely reversible in the presence of high concentrations of cationic surfactants (Ericsson et al., 1983a). This was explained by decreased inter- and intramolecular interactions at high temperature, due to interaction between the unfolded protein and surfactant, which facilitates the re-formation of the native complex on cooling.

As a rule of thumb, an increase in pH will shift the binding of anionic surfactants to higher concentrations (Reynolds et al., 1970). In this case one would expect that both the specific and the cooperative binding are affected in the same way. A decrease of pH will have the same effect on binding of cationic surfactants (Subramanian et al., 1984). At low surfactant concentrations, that is, well below cmc, cationic amphiphiles increase the solubility of proteins on the acidic side of the isoelectric point (pI), while precipitation can occur on the alkaline side of pI. Anionic amphiphiles will affect solubility in the opposite direction. The solubilizing effect is also observed at high temperatures.

We conclude that since the binding generally is thought to occur via monomers, any change affecting the cmc will also affect the cooperative binding at concentrations close to and above cmc. Under some conditions the formation of surfactant micelles will be energetically favored compared to binding to the protein. If cmc is of the same order of magnitude as the concentration necessary for binding to occur, the lowering of cmc caused by increasing ionic strength might even prevent binding.

5.3.2 *Phase Behavior of Emulsifier Protein Systems*

So far we have mainly addressed the interaction at low protein concentrations. Morén and Khan (Morén and Khan, 1995) investigated the phase behavior of the anionic SDS, positively charged lysozyme and water over a wide concentration range and one of the phase diagrams they determined is given in Fig. 5.7a. Stenstam et al. later investigated in detail the stoichiometry of the formed complex and their findings are summarized in Fig. 5.7b (Stenstam et al., 2001). Small amounts of SDS, at a ratio to lysozyme corresponding to charge neutralization of the protein, were found to give precipitation. A net attractive force exists between the surfactant–protein complexes and hydrophobic interactions dominate (Fig. 5.7b). Further addition of SDS dissolved the precipitate and complete dissolution was achieved when the number of SDS molecules was equal to the number of (18) positive charges on the protein. A bluish gel phase occurred when the protein concentration was between 7–20% (w/w). A further increase of the ratio between SDS and lysozyme, leads to a strong net repulsive electrostatic interaction between the surfactant–protein complexes (Fig. 5.7b). Consequently an isotropic solution is formed. Morén and Khan also investigated the effect of varying alkyl chain length, $C_{12}SO_4$, $C_{10}SO_4$, C_8SO_4, and C_6SO_4 on the lysozyme –sodium alkyl sulfate-water ternary systems (Morén and Khan, 1998). The extension of the solution region decreased with increasing surfactant chain length and the surfactant with shortest hydrophobic tail (C_6SO_4) forms the largest solution region with lysozyme without precipitation. The extension of the precipitation region toward higher surfactant concentrations increases with decreasing

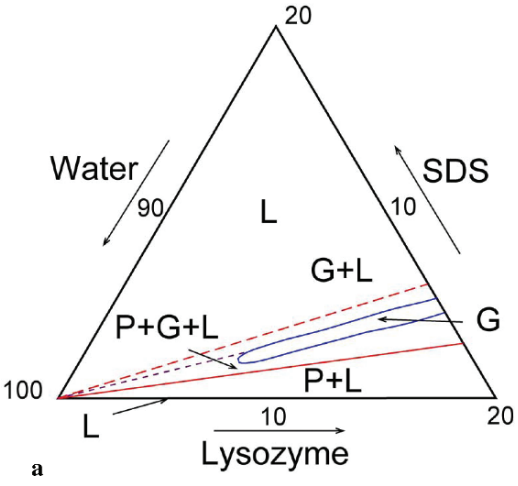

Fig. 5.7a Phase diagram of the lysozyme–SDS–water ternary system, where L indicates solution, G gel and P precipitate. The figure is adapted from (Morén and Khan, 1995), where experimental details are given

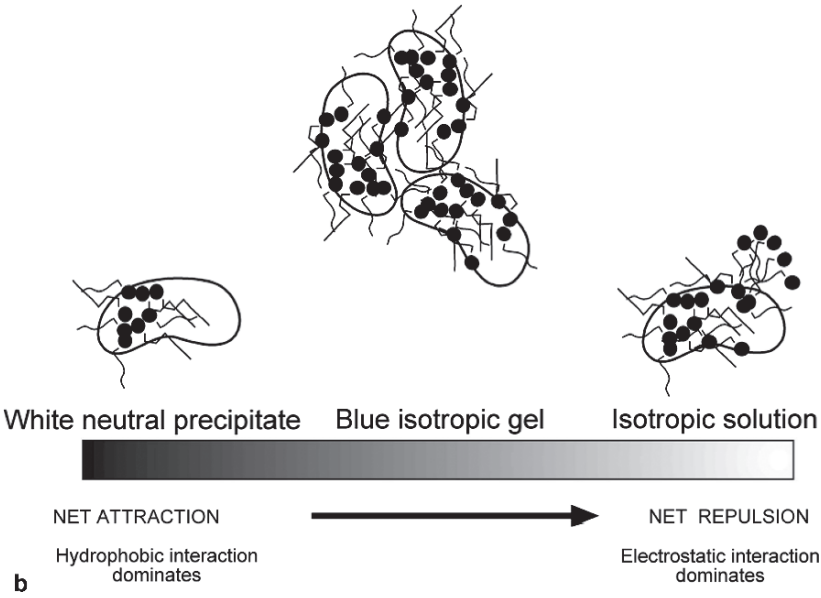

Fig. 5.7b Schematic representation of the interaction between protein surfactant complexes in the lysozyme–SDS–water system. Figure adapted from Stenstam et al. (2001), where the experimental details are given

surfactant chain length. The surfactant concentration required to redissolve the precipitate at dilute protein concentrations therefore seems to follow the cmc for the surfactant in water, which also increases with decreasing surfactant chain length. A single gel phase was only observed for the $C_{12}SO_4$ and $C_{10}SO_4$ systems and not in presence of C_8SO_4 and C_6SO_4. Similar types of gel phases are expected to occur in more food relevant surfactant/lipid and protein aqueous mixtures and therefore offer interesting possibilities to vary the functional properties of foods and food ingredients.

5.3.3 Emulsifiers with Low Aqueous Solubility

For emulsifiers with low aqueous solubility the emulsifier self-assembly structure and its properties control the interaction with proteins

In this section we will discuss interactions involving lipids with low solubility where the lipids exist as dispersed particles, liposomes or vesicles, liquid crystalline phases as well as monolayers at interfaces. Many of the principles discussed in the earlier sections, also do apply for protein–lipid interactions in condensed systems.

Polar lipids, which normally are water-insoluble, associate into a variety of structures in aqueous solution. This process will have an impact on interactions with proteins. For lipids with low aqueous solubility the interaction with the proteins mainly involves the self-assembled structure formed by the lipids. However, we note that even polar lipids that are considered water-insoluble have a certain monomer solubility, which although small (about 10^{-7} for monoolein and about 10^{-10}–10^{-12} M for phospholipids) makes it possible for them to interact with proteins in the monomeric form, in particular if the protein has a high affinity binding-site for the lipids. This is demonstrated in Fig. 5.8, which shows the thermograms from differential scanning calorimetry measurements of β-lactoglobulin, distearoylphosphatidic acid (DSPA) and β-lactoglobulin + an aqueous dispersion of DSPA, respectively. The peak corresponding to the thermally induced unfolding transition of β-lactoglobulin in presence of DSPA is shifted towards higher temperature compared to the one recorded for the pure protein. This confirms the presence of a specific interaction between phosphatidic acid and β-lactoglobulin that thermally stabilizes the protein. This was also observed in the presence of dipalmitoylphosphatidic acid (DPPA), but no such interaction was observed when the protein was mixed with phosphatidylcholine, phosphatidylethanolamine or phosphatidylglycerol (Kristensen et al., 1997). Neither could any interaction be observed if the lipid contained unsaturated fatty acid residues. Thus the results show that the interactions between β-lactoglobulin

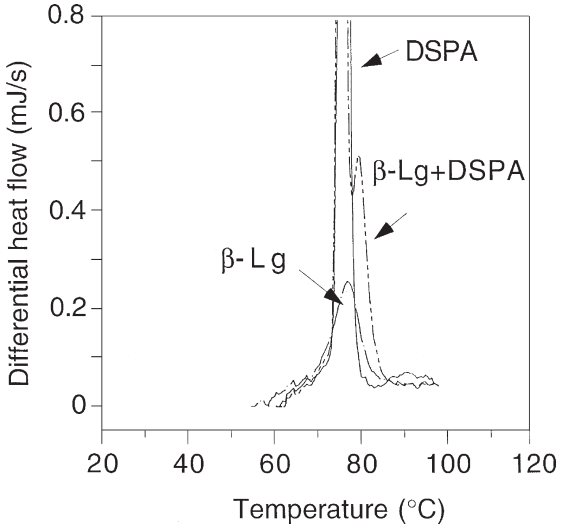

Fig. 5.8 The interaction between distearoylphosphatidic acid (DSPA) and β-lactoglobulin (β-Lg) is demonstrated by the results from differential scanning calorimetry (DSC) where the thermogram of the protein/lipid mixture is compared with those of the pure components. The thermograms of DSPA, 5% (w/v) (——), β-Lg 5% (w/v) (— . —) and a mixture of β-Lg 5% and DSPA 5% (w/v) (— - - —) in 1% sodium chloride at pH 7. A scanning rate of 10 °C/min was used. Data adapted from Kristensen et al. (1997), where also the experimental details are given

and phospholipids are strongly dependent on the acyl chain as well as the head group. A small negatively charged head group is needed for the interaction to take place. Such an interaction can have important implications for the functional properties of the protein. We discussed above that fatty acids bound to β-lactoglobulin could affect the interfacial behavior of the protein (Clark et al., 1995). Kurihara and Katsuragi reported that a lipid–protein complex, formed between β-lactoglobulin and phosphatidic acid, could mask bitter taste (Kurihara and Katsuragi, 1993). This property was suggested to be specific for phosphatidic acid as no effect was observed for mixtures of β-lactoglobulin and phosphatidylcholine, triacylglycerol or diacylglycerol.

Even if no specific interaction occurs, proteins can have an impact on liquid crystalline phase or gel phase due to the limited space of the aqueous cavity. This was demonstrated by Minami et al, who investigated the incorporation of lysozyme, β-lactoglobulin and α-lactalbumin in a sphingomyelin gelphase containing 0.6 wt% sodium palmitate and 80 wt% aqueous solution (Minami et al., 1996). The dimension of the aqueous layer in the gel phase was suggested to limit the amount of protein that could be incorporated. Above this limit, phase separation will occur with a gel phase and an "outside" protein rich solution. The protein will, at high enough concentration, probably also compete for the water in the interlamellar spacing, which eventually leads to a reduction of the aqueous layer thickness. This effect was demonstrated for high molecular weight polymers in equilibrium with the phosphatidylcholine lamellar phase (LeNeveu et al., 1977). The polymer was unable to enter the aqueous layer, but still exerted an osmotic stress that was large enough to compress the lamellar lattice as shown by x-ray diffraction data. This method has been used to measure the interaction between the lipid bilayers (LeNeveu et al., 1977; Cowley et al., 1978).

Proteins are of course also able to enter into the aqueous layer of a lamellar phase and thereby affect the swelling. This was shown by Rand (Rand, 1971), who studied the penetration of bovine serum between negatively charged lecithin-cardiolipin mixed bilayers in a lamellar phase at pH 3.3, where the protein has a positive net charge. BSA is also likely to adopt a more expanded structure at this pH, thus exposing more hydrophobic segments. He found that the inter-lamellar spacing of the lamellar phase, decreased with decreasing cardiolipin/bovine serum albumin ratio. This was related to a reduction of the negative charge of the lipid layer as the amount of bound protein increases.

We will start our discussion by giving some example of the interplay between the lipid structures and protein in terms of the effect on the curvature of the lipid-aqueous interface, since curvature place an important role in condensed matter as discussed in the book by Hyde et al. (Hyde et al., 1997).

5.3.3.1 Protein Interactions that Increase the Curvature of the Lipid-Aqueous Interfaces

Proteins or peptides that penetrate into the hydrophobic domain of a lipid bilayer generally provokes an increase of curvature of the lipid-aqueous interface, i.e., becomes

more concave towards the aqueous space. Quite a few of the membrane bound peptides have these properties, such as Gramicidin A, a hydrophobic polypeptide, which forms channels for monovalent cations in phospholipid membranes (Wallis, 1986). This peptide was found to favor the transition between lamellar phase → reversed hexagonal (H_{II}) phase in dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE) systems in an excess of water, as observed by NMR-studies (Chupin et al., 1987).

Not only proteins or peptides that penetrate into the lipid bilayer can induce phase transitions, but also proteins that mainly interact with the headgroups of the phospholipid bilayer can give rise to similar effects. This has been demonstrated for cytochrome c, which has a positive net charge and has been shown to interact with negatively charged phospholipids (De Kruijff and Cullis, 1980). The binding of cytochrome c to anionic cardiolipin liposomes induced the formation to an inverted hexagonal, H_{II}, structure (De Kruijff and Cullis, 1980). No interaction and hence no phase transition was observed in the presence of liposomes composed of neutral zwitterionic lipids like PC and PE. A phase transition to the H_{II}-phase was observed, if a sufficient fraction of these lipids was replaced for cardiolipin. Interestingly, the protein was found to interact with liposomes of the anionic lipid phosphatidylserine (PS), but did not induce any phase transition. The interaction between cardiolipin and cytochrome c was also studied by Spooner and Watts, using deuterium and phosphorus 31 NMR measurements (Spooner and Watts 1991b). They likewise found that the interaction can, depending on the lipid stoichiometry, cause a transition from a lamellar to a nonbilayer structure. The binding of the protein with the liquid-crystalline bilayers of cardiolipin was also found to cause extensive rearrangement of the cytochrome c secondary structure (Spooner and Watts, 1991b; Spooner and Watts, 1991a).

Studies of the interaction between cytochrome c and suspensions of DMPG or admixtures of dioleoylglycerol (DOG) or DOPC with DOPG also showed that binding of cytochrome c could promote an increase in surface curvature of the lipid aggregates from a bilayer structure (Heimburg et al., 1991). This is deduced from NMR-data where an isotropic peak occurs in the presence of cytochrome c, indicating cubic lipid phases, small spherical vesicles or extended bilayers with high local curvature. The structure of cytochrome c was found to change on binding to the lipid, and two forms, depending on the lipid composition, were identified with resonance Raman measurements:

I. close to the native conformation in solution
II. unfolded with the heme crevice opened

The changes in protein structure could be correlated with the curvature of the lipid bilayer as illustrated in Fig. 5.9 as the ratio between the unfolded (II) and native (I) cytochrome c (cyt c) in DOPC/DOG dispersions versus DOG mol %. The presence of DOG was found to induce spontaneous curvature in the DOPG lipid bilayer in the pure lipid system, which at DOG content of ≈50% leads to the transition to a reversed hexagonal (H_{II}) phase. In the absence of DOG, that is a strict bilayer structure, the binding of the more unfolded form (II) of cytochrome is favored, whereas the fraction of the more native globular protein structure (I) increases with the

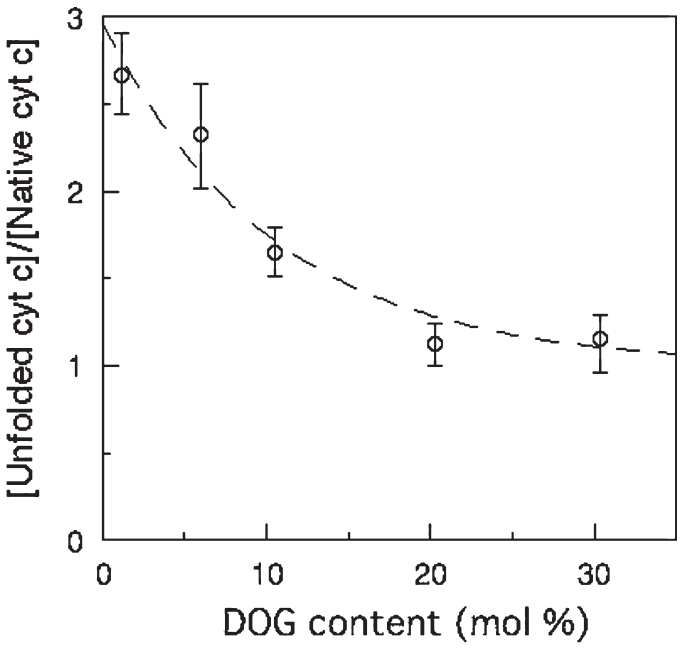

Fig. 5.9 Concentration of unfolded (II) and native (I) cytochrome c (cyt c) in dioleoylphosphatidylcholine (DOPC)/dioleoylglycerol (DOG) dispersions versus DOG mol% determined from Raman resonance spectra. The concentrations of lipid and cytochrome c were 300 and 20 μM, respectively, in an aqueous buffer (1-mM Hepes, 1-mM EDTA) of pH 7.5. Data adapted from Heimburg et al. (1991), where also the experimental details are given

amount of DOG (Fig. 5.9) and thus with curvature of the surface. The physical state of the lipid was also found to affect the proportions of the two structural forms of cytochrome c. In the fluid state of pure DMPG, the fraction of the more unfolded form (II) was larger (85%) than when the lipid was in the gel state (80%). It is noteworthy that they found that the bound fraction of the more unfolded form (II) to the fluid DOPG bilayer structure was substantially lower (75%), indicating that not only the fluidity of the bilayer matters, but also the type of lipid.

The interaction between cytochrome c and monoolein in the cubic phase was studied by Razumas et al. by differential scanning calorimetry (DSC) and optical microscopy (Razumas et al., 1996a). In line with the studies reported above they also found that the presence of cytochrome c at high enough concentrations favored lipid aggregates with a larger curvature. Thus they observed that the phase transitions cubic $\rightarrow H_{II} \rightarrow L_2$ in the monoolein-cytochrome c-water system took place at a lower temperature than in the binary monoolein-water system (Razumas et al., 1996a). Similar effects were observed when glucose oxidase was included into monoolein-aqueous cubic phase (Barauskas et al., 2000). The temperature of the phase transition cubic $\rightarrow H_{II}$ in the monoolein-glucose oxidase aqueous system decreased with increasing glucose oxidase concentration.

5.3.3.2 Protein Interactions that Decrease the Curvature of the Lipid-Aqueous Interfaces

McCallum and Epand found that changing the curvature of biological membranes could modify membrane bound insulin receptor autophosphorylation and signaling (McCallum and Epand, 1995). This was demonstrated by adding compounds that raised the bilayer to reverse hexagonal (H_{II}) transition temperature of model membranes, that is decrease the curvature of the mebrane. This inhibited the insulin stimulation of the receptor phosphorylation.

Fraser et al. investigated the ability of a range of basic proteins and polylysine to convert a reversed hexagonal (H_{II}) phase, consisting of dioleoylphosphatidylethanolamine (DOPE) and mixtures of DOPE and phosphatidylserine (PS), to stable lamellar (L_{α}) phases at pH 9 where DOPE is anionic and at pH 7 when it is zwitterionic (Fraser et al., 1989). The proteins investigated were all capable of binding to the H_{II}-phase at pH 9, but only myelin basic protein and polylysine did induce transition to the L_{α}-phase. Lysozyme formed a new H_{II}-phase where the protein was included. A lowering of the pH seemed to release the proteins, except for mellittin, which also seemed to penetrate into the hydrophobic core of the lipid aggregates. The presence of PS in the H_{II}-phase at pH 7 increased the protein binding, but only interaction with myelin basic protein gave a lamellar phase. Based on earlier studies, Fraser et al. suggested that the myelin basic protein stabilized the lamellar phase by interacting with the DOPE headgroup and thereby increasing its effective size (Fraser et al., 1989). They concluded that the properties of myelin basic protein in terms of stabilizing the lamellar structure could be related to the role of the protein to stabilize the myelin sheath multilayers.

5.4 Interaction between Protein and Surfactants or Polar Lipids at Interfaces

Defining different plausible scenarios and principles and defining simple models

Interactions between proteins and surfactants at air/water and oil/water interfaces has attracted considerable study in recent years because the consequences of competitive adsorption of these two species at these interfaces can often strongly influence dispersion (foam or emulsion) stability against coalescence. The majority of proteins have high affinity for interfaces, which they saturate at comparatively low concentrations compared to low molecular weight (LMW) surfactants (Dickinsson and Woskett, 1989; Coke et al., 1990). Thus, on a mole for mole basis at low concentrations, proteins reduce the surface tension to a greater extent than LMW surfactants. However, the opposite effect is observed at high concentrations, because at saturation coverage with LMW surfactants, the interfacial tension of the interface is usually lower than that achieved by proteins, and as a result, the latter molecules will be displaced from the interface. The region where the two different components coexist in the interfacial layer is of greatest interest, since it is in this region that will mostly affect the stability of the system towards coalescence.

The mechanisms by which proteins, polar lipids or mixtures of them stabilize emulsions and foams can be quite different. Generally, polar lipids are capable of reducing the interfacial tension more than proteins, while the protein molecules can be anchored at multiple sites at the interface. In principle thin films are stabilized by two distinct mechanisms; the one that dominates is dependent upon the molecular composition at the interface (Clark, 1995). Low molecular weight surfactants such as food emulsifiers or polar lipids congregate at the interface and form a fluid-adsorbed layer at temperatures above their transition temperature (see Fig. 5.10). When a surfactant-stabilized thin film is stretched, local thinning can occur in the thin film. This is accompanied by the generation of a surface-tension gradient across the locally thin region. Surface tension is highest at the thinnest point of the stretched film, due to the local decrease in the surface concentration of emulsifier in the region of the stretch. Equilibrium surface tension is restored by adsorption of surfactant from the interlamellar liquid, which is of very limited volume in a drained thin film. This process is called the "Gibbs effect." Alternatively, migration of the surfactant by lateral diffusion in the adsorbed layer toward the region of highest surface tension may also occur (Clark et al., 1990b). Here, the surfactant drags interlamellar liquid associated with the surfactant head group into the thin region of the film and contributes to the restoration of equilibrium film thickness. This process is often referred to as the "Marangoni effect" (Ewers and Sutherland, 1952).

In contrast, the adsorbed layer in protein-stabilized thin films is much stiffer and often has viscoelastic properties (Castle et al., 1987). These derive from the protein/protein interactions that form in the adsorbed layer (see Fig. 5.10b). These interactions result in the formation of a gel-like adsorbed layer, referred to as a "protein-skin" (Prins, 1999), in which lateral diffusion of molecules in the adsorbed layer is inhibited (Clark et al., 1990a). Multilayer formation can also occur and serves to further mechanically strengthen the adsorbed layer (Coke et al., 1990). When pure protein films are stretched, the change in interfacial area is dissipated across the film, due to the cohesive nature of the adsorbed protein layer and possibly the deformability of the adsorbed protein molecules.

Thin-film instability can result in systems that contain mixtures of proteins and low molecular weight surfactants (Coke et al., 1990; Clark et al., 1991b; Sarker et al., 1995), as is the case in many foods. The origin of this instability rests in the incompatibility of the two stabilization mechanisms: the Marangoni mechanism relying on lateral diffusion, and the viscoelastic mechanism on immobilization of the protein molecules that constitute the adsorbed layer. One can speculate that in a mixed system, competitive adsorption of low molecular weight surfactant could weaken or interfere with the formation of protein/protein interactions in the adsorbed layer and destroy the integrity and viscoelastic properties of the adsorbed layer (see Fig. 5.10c). This could be a progressive process, with the presence of small quantities of adsorbed surfactant initially introducing faults or weaknesses in the protein film. Adsorption of more surfactant could induce the formation of protein "islands" in the adsorbed layer. These structures could be capable of slow lateral diffusion but would be too large to participate in Marangoni-type stabilization. Indeed, they could impede surfactant migration in the adsorbed layer. Adsorption of progressively more surfactant would reduce the size of the protein aggregates still further until the adsorbed protein was in its monomeric form. Ultimately, all the protein would be displaced from the interface by the surfactant.

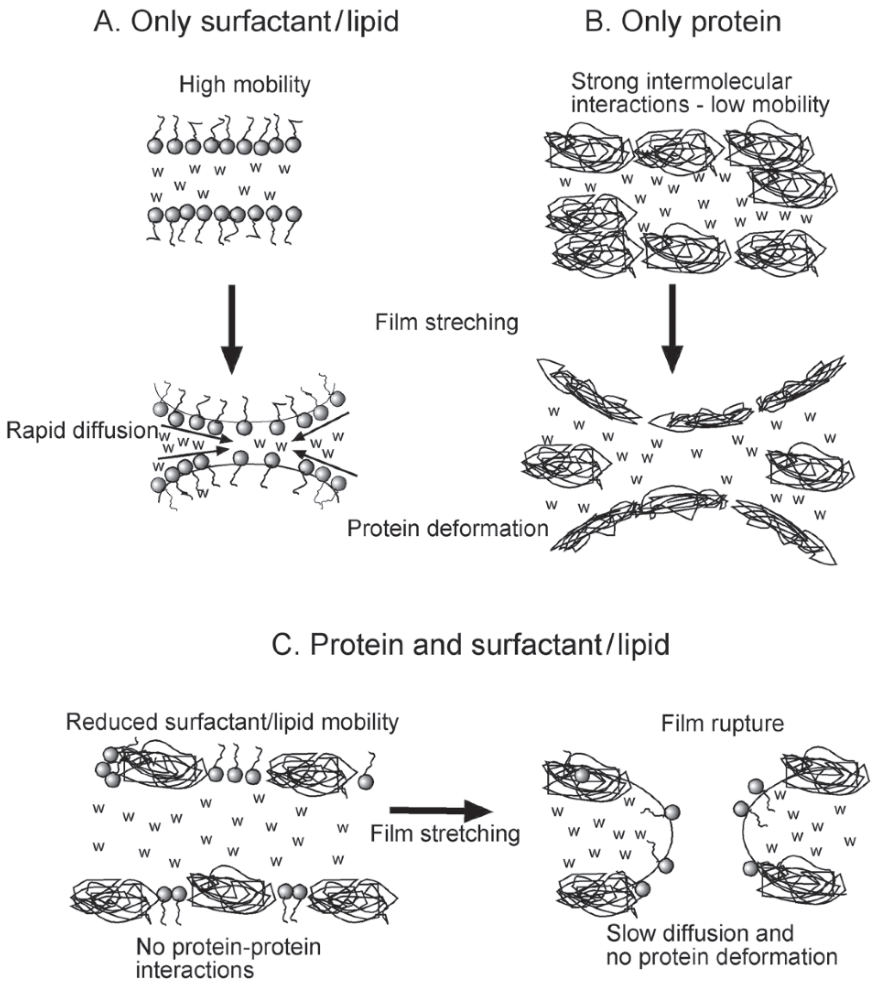

Fig. 5.10 The figure depicts possible mechanism for the stabilization–destabilization of foams with surfactants/lipids (A), proteins (B) and mixtures of the two components (C). Cross-sections of the thin films are shown where the aqueous inter-lamellar spacing is marked with (w). The stabilization of the surfactant/lipid foams are based on the high lateral mobility of the surfactant, which makes it possible to quickly restore the surface tension gradient which arises from thinning of the film, i.e., the Gibbs-Marangoni effect. For protein stabilized foam the thinning is counteracted by strong intermolecular interactions which give a viscoelastic film. For the mixed system two mechanisms can counteract each other and leads to film rupture. The figure is adapted from Clark et al. (1991a)

Two types of interaction are shown in the schematic diagram of the mixed system. First, there is an interactive process associated with the coadsorption or competitive adsorption of the two different species at the interface. Second, many of the functional proteins used in food production have physiological transport roles and therefore posses binding sites, which may allow the formation of complexes with surfactants.

A c1earer understanding of this has emerged from direct study of the structures that separate the dispersed-phase of foams or emulsions, under conditions of high

dispersed phase volume (i.e., foam or emulsion thin films). Such structures form rapidly in foams following limited drainage but may occur only in emulsions after creaming of the dispersed phase.

Several factors control the emulsifier-protein interaction at the interface

On the bases of experimental data the following factors influence the way mixtures of proteins and emulsifiers, e.g., surfactants and polar lipids behave at an interface:

1. The surface activity of the individual components.

a. Competitive adsorption.

The emulsifier and proteins compete for the interface, where the most surface active and/or abundant molecule wins, depending on the ratio between surfactants and proteins in solution.

b. Displacement.

The emulsifier may, due to their higher surface activity, displace the proteins from the interface. This displacement can be hampered by a strong interaction between the protein and the interface and/or protein-protein interactions.

2. Protein-emulsifier interactions.

Increased surface activity of the emulsifier-protein complex

(a) The binding will cause unfolding and/or increase hydrophobicity of the protein that will lead to an increased affinity to the surface.
(b) The binding (of ionic amphiphiles) will cause precipitation at the interface due to charge neutralization.

Decreased surface activity of the emulsifier-protein complex

(a) The binding will make the protein more soluble and hence lower the affinity for the interface.
(b) The binding will lead to precipitation of protein lipid-complex in the bulk, which will cause loss of surface-active material.

Protein- emulsifier interactions at the interface

(a) The interaction will give more efficient packing at the interface and thus give a higher total surface concentration.
(b) The interaction will disrupt the protein-protein interaction in the interfacial film.

It is important to bear in mind that different modes of interaction are observed for the same system depending on the emulsifier/protein ratio. This can be for instance is manifested in the competitive adsorption of emulsifier and proteins. Studies regarding such surfactant/protein "Vroman effects"[3] have been reported; for example,

[3] The "Vroman effect" is the hierarchical adsorption process of blood protein, where the first proteins to be adsorbed are the relatively abundant plasma proteins, such as albumin, fibrinogen, immunoglobulin G and fibronectin, which are soon replaced by trace proteins, including factor XII (Hageman factor) and high molecular weight kininogen (HMWK) with higher affinity to the surface (Vroman et al., 1980; Brash and Hove, 1984; Horbett, 1984).

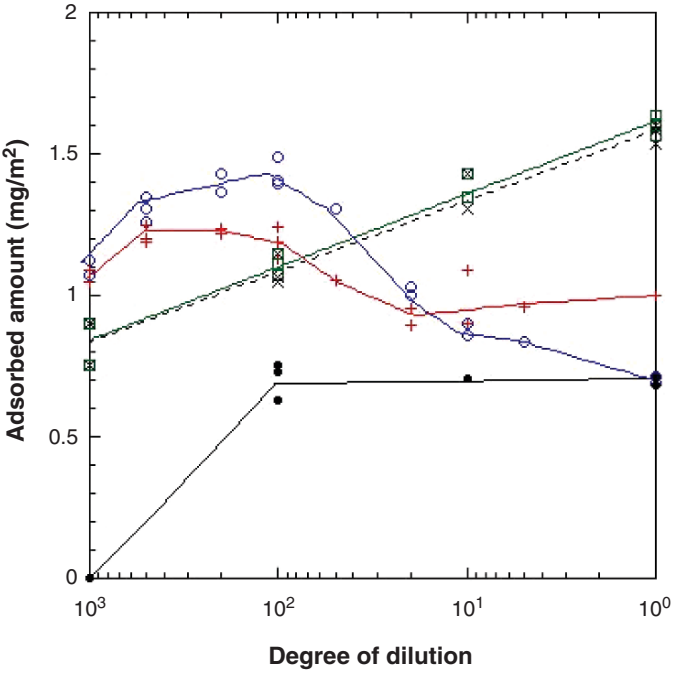

Fig. 5.11 The amounts adsorbed to a methylated silica surface as a function of degree of dilution for a mixture of β-lactoglobulin and SDS (0.2 w/w), in phosphate buffered saline pH 7, I = 0.17. The figure shows the adsorbed amount (μg/cm²) after 30 min of adsorption (○) and 30 min after rinsing (+). In addition, the figure shows the adsorption of pure β-lactoglobulin, after 30 min of adsorption (❑) and 30 min after rinsing (x). Finally, the adsorption isotherm of SDS is inserted (●). Adapted from Wahlgren and Arnebrant (1992)

adsorption of fibrinogen from mixtures containing Triton X-IOO passes through a maximum (Slack and Horbett, 1988). Wahlgren and Arnebrant studied the adsorption from β-lactoglobulin/SDS mixtures at different degrees of dilution (Wahlgren and Arnebrant, 1992) (see Fig. 5.11). At concentrations above the cmc for the surfactant, the amount adsorbed corresponded to a layer of pure surfactant and was found to increase after rinsing. At lower concentrations, the adsorbate prior to rinsing appeared to be a mixture of protein and surfactant, and the total amount adsorbed passes through a maximum. The amount of protein adsorbed is larger, even after rinsing, than for adsorption from pure β-lactoglobulin solutions, and it can be concluded that SDS binding in this case facilitates the adsorption of protein.

5.4.1 Influence of Emulsifier Properties

The emulsifier properties affect the interaction with proteins and surfaces as well as the structure of the formed self-assembled aggregate.

5.4.1.1 Aqueous Soluble–Surfactant Type of Emulsifiers

Wahlgren and coworkers studied the influence of different surfactant head groups on the desorption of adsorbed lysozyme (Wahlgren and Arnebrant, 1991; Wahlgren and Arnebrant, 1992; Wahlgren et al., 1993b) by surfactants at concentrations above the cmc (an exception was triethylene glycol n-dodecyl ether, [$C_{12}E_3$, which does not form micelles (Mitchell et al., 1983)]). The difference between the effect of sodium dodecylsulphate (SDS) and cationic and nonionic surfactants on protein adsorption to hydrophilic surfaces was found to correlate to the strength of binding to protein in solution. This suggests that above the critical association concentration (cac), complex formation between surfactant and protein is involved in the removal mechanism of proteins from hydrophilic solid surfaces. In the case of hydrophobic solid surfaces, the removal processes of protein by the different surfactants, including non-micelle-forming ones, are in general more similar than for the hydrophilic surfaces. This might be expected, due to the different orientation of the surfactant, and suggests a displacement mechanism, due to higher surface activity of the surfactant (Wahlgren and Arnebrant, 1992). Tilton and coworkers used the interferometric surface force technique (Israelachvili and Adams, 1978) to study the interaction between lysozyme adsorbed on mica and SDSo (sodium dodecane sulfonate) and SDS (Tilton et al., 1993). They found that SDSo, which has a Krafft temperature above room temperature and hence does not form micelles, had a minor effect on the interaction between adsorbed lysozyme layers on mica, and from the small change in surface potential, they concluded that few surfactant molecules were bound to the adsorbed protein. SDS showed a similar low binding to lysozyme on mica at low concentrations (up to 0.5 cmc) but caused a collective desorption of the protein at the cmc of the surfactant, indicating that the cac to adsorbed lysozyme is in the range of its self-association limit in solution (cmc) (Froberg et al., 1999). These studies show that anionic surfactants bind to an adsorbed layer of lysozyme, which is almost neutral after binding of the positively net charged protein to the negative mica surface. The binding of surfactant thus leads to an increased negative charge of the layer, which in the case of SDS finally leads to desorption of the protein. It is likely that this is due to electrostatic repulsion between the negatively charged surface and the protein/surfactant complexes.

Nonionic surfactants are generally found to be ineffective in removing protein from hydrophilic solid surfaces (Elwing et al., 1989; Elwing and Golander, 1990; Welin-Klintström et al., 1993). As mentioned above, these surfactants bind to a very low extent to protein in solution (except when specific binding sites or pockets are present) and to the protein-covered surface. At hydrophobic surfaces, however, they usually have a considerable effect (Wahlgren and Arnebrant, 1996; Wannerberger et al., 1996). This was elegantly demonstrated in a study of surfactant interactions with proteins adsorbed at a surface with a gradient in wettability (Elwing et al., 1989).

The effect of chain length of alkyltrimethylammonium surfactants on the elutability of fibrinogen at concentrations above their cmc was found to be small at both silica and methylated silica surfaces (Wahlgren et al., 1993a). Rapoza and Horbett (1990a) did not find any effects of chain length of sodium alkyl sulfates on the

elutability of fibrinogen and albumin down to a chain length of 6 carbon atoms. However, they found, as expected, that the chain length did influence the surfactant concentration at which the onset of protein removal was initiated. The trend was similar to the one observed for the onset of other cooperative binding events (e.g., micelle formation).

Rapoza and Horbett (1990b) found that surfactants with large head groups such as Tween 20 gave lower fibrinogen elutability levels than other surfactants at polyethylene surfaces. Welin-Klintström et al. (1993) found that the elutability of fibrinogen adsorbed at surfaces with a wettability gradient decreased with the bulkiness of the hydrophobic part of the surfactant. In this connection it was also found that nonionics showed an increased removal of fibrinogen into the more hydrophilic region of the gradient surface when the cloud point (phase separation temperature) was approached (Wahlgren et al., 1995). These general observations of removal efficiency are in line with the findings from studies of the removal of fat by different surfactants (Backstrom et al., 1988; Malmsten and Lindman, 1989), where a maximum removal was achieved at conditions corresponding to an optimum in the packing of surfactant molecules at a flat interface.

Thus, it may be concluded that at high surfactant concentrations, head group effects are, as expected, most pronounced at hydrophilic surfaces but less important at hydrophobic ones. In addition, it appears that principles for detergency in general, involving the packing efficiency of molecules at interfaces, are applicable to qualitatively describe the removal of proteins from the surface.

5.4.1.2 Lipids with Low Aqueous Solubility

Electrostatics

Phospholipid - β-lactoglobulin interactions at the air - aqueous interface have been investigated by Bos and Nylander (Bos and Nylander, 1995) using the surface film balance. Some of their findings are summarized in Fig. 5.12, where the rate of incorporation of β-lactoglobulin into monolayers of distearoylphosphatidic acid (DSPA), distearoylphosphatidylcholine (DSPC) and dipalmitoylphosphatidic acid (DPPA) is shown versus surface pressure (Π) at pH 7. The rate was calculated using a simple first order kinetics model (MacRitchie, 1990), where only the surface pressure barrier is taken into account. The highest rate of adsorption of β-lactoglobulin into a phospholipid monolayer was observed for anionic DSPA. The incorporation of the protein takes also place at a higher surface pressure into a DSPA monolayer than into a monolayers of the other lipids. Since the β-lactoglobulin, with a zero net charge at pH 5.2 (Hambling et al., 1992), has a positive net charged at pH 4, a larger rate of adsorption into the negatively charged phosphatidic acid monolayers would be expected under acidic conditions. However, almost the same rates were found (Bos and Nylander, 1995). As discussed earlier, anionic lipids seems to interact more strongly with proteins, that is to their cationic amino acid residues, compared to lipids with none or positive net charge. The incorporation into the zwitterionic DSPC

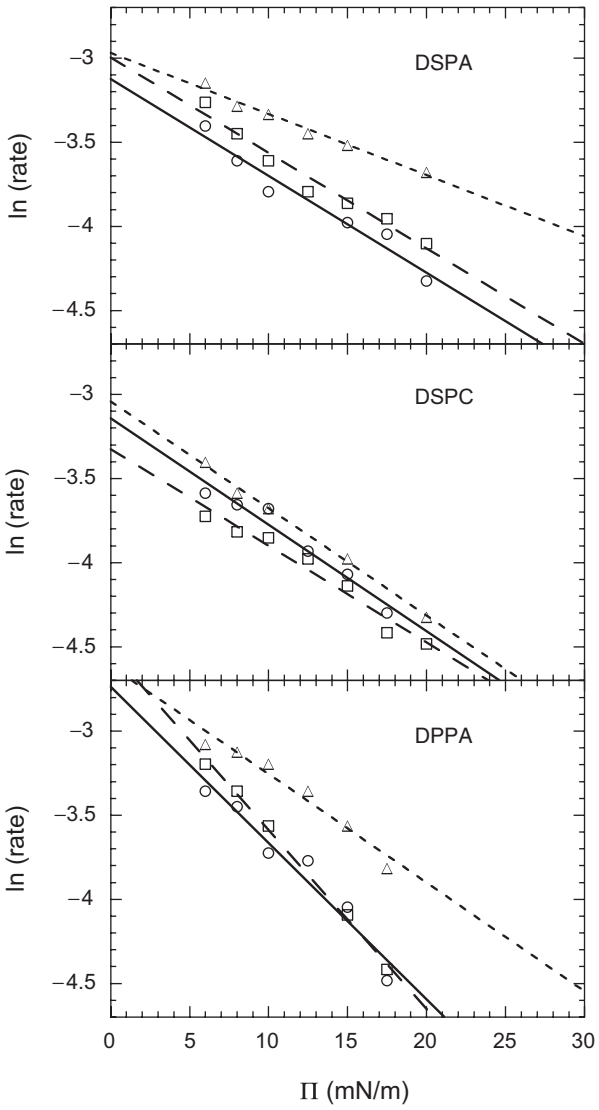

Fig. 5.12 The rate of incorporation of β-lactoglobulin into monolayers of distearoylphosphatidic acid (DSPA), distearoylphosphatidylcholine (DSPC) and dipalmitoylphosphatidic acid (DPPA), versus surface pressure (Π). The data was recorded at constant surface pressure by measuring the area increase of the lipid monolayer spread on a protein solution contain 1.15 mg/l in 10 mM phosphate buffer of pH 7, with 0 mM (— ❍ —), 50 mM (—— ❑ ——) or 150 mM (- - - Δ- - -) sodium chloride. The rate in mg/m^2 was calculated from the area increase by using the Π-area isotherm of spread monolayers of β-lactoglobulin. Data adapted from Bos and Nylander (1995), where also the experimental details are given

monolayers is as expected less salt dependent than what was observed for the phosphatidic acid monolayers, where the rate increases with increasing ionic strength of the subphase. Probably this is a consequence of a decreased repulsion within the phosphatidic acid protein monolayer at a higher ionic strength. The findings by Bos and Nylander (Bos and Nylander, 1995) is somewhat contradictory to the findings of Cornell and Patterson, who studied the adsorption of β-lactoglobulin in to a negatively charged lipid monolayer, composed of a mixture of palmitoyloleoylphosphatidylcholine (POPC) and palmitoyloleoylphosphatidylglycerol (POPG) (65/35 mol %). They only observed a substantial binding of β-Lactoglobulin at pH 4.4, which is when the protein carries a net positive charge, but not at higher pH (pH 7) (Cornell and Patterson, 1989). The differences probably arises from the different lipids and methodology used by Cornell et al.(Cornell, 1982; Cornell and Patterson, 1989; Cornell et al., 1990). Cornell et al. measured the amounts of protein adsorbed to the lipid layer by transferring the layer to a solid support. During the transfer, the surface pressure was kept at 30–35 mN/m, thus preventing insertion of portions of the protein in the lipid monolayer (Cornell et al., 1990). Only protein molecules that interact strongly with the lipid headgroups are transferred to the solid supported. Another difference is that their surface pressure data of the protein penetration is recorded under constant area, not at constant pressure as in our study. In addition Cornell et al. used lipids with their chains in the liquid state, which, as discussed below, can influence the interaction. Cornell (Cornell, 1982) also observed a specific interaction between β-lactoglobulin and egg yolk phosphatidic acid (e-PA) in spread mixed films at low pH (1.3 and 4) where β-lactoglobulin carries a positive net charge. No interaction was observed for e-PA in the neutral pH range or for egg yolk phosphatidylcholine, e-PC. Similar observations were made for the interaction between α-lactalbumin or BSA with mixed monolayers of POPC and POPG, where adsorption was observed below the isoelectric point of the protein, where the lipid layer and the protein carry opposite net charge, but less was adsorbed around and almost nothing above the isoelectric point (Cornell et al., 1990). The interaction was reduced in the presence of calcium as well as at increased ionic strength. Cornell et al. thus concluded that the interaction is of electrostatic origin.

The work of Quinn and Dawson concerning the interaction between cytochrome c (positive net charge below pH 10) and phospholipids from egg yolk also stresses the importance of the electrostatic interaction, although conformational changes of the protein are of importance (Quinn and Dawson, 1969b; Quinn and Dawson, 1969a). They measured the pressure increase caused by the penetration/adsorption of the protein to the lipid monolayers as well as the amount adsorbed by using ^{14}C-labeled protein. Their results show that the limiting pressure for penetration is 20 and 24 mN/m for phosphatidylcholine and phosphatidylethanolamine, respectively, whereas penetration into the phosphatidic acid and diphosphatidylglycerol (cardiolipin) monolayers occurred up to pressures close to the collapse pressure of the film (< 40 mN/m). Furthermore, the penetration into the e-PC monolayers was not affected by increasing the sodium chloride concentration to 1 M. Cytochrome c bound to the e-PC monolayers could not be removed by increasing the ionic strength. This is in contrast to the cardiolipin and e-PA monolayers where the penetration was

reduced when the sodium chloride content was increased to 1 M. It was also possible to partly desorb some cytochrome c from e-PA monolayers. However, the pH dependence of the interaction was found to be quite complex, which suggests that subtle changes in the protein conformation also affect the interaction.

The importance of the electrostatic interaction with the phospholipid head group has also been shown by the work of Malmsten (Malmsten et al., 1994; Malmsten, 1995), who studied the interaction of human serum albumin, IgG and fibronectin from human plasma with phospholipid layers spin-coated onto methylated silica surfaces. Generally, he found no interaction between the proteins and lipids with no net charge or with shielded charges (e.g., phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphatidylinositol), whereas interaction was observed with the surfaces containing unprotected charges, e.g., phosphatidic acid, diphosphatidylglycerol and phosphatidylserine.

Hydrophobic Interactions

As observed in Fig. 5.12 the rate of adsorption of β-lactoglobulin into DPPA monolayers was significantly lower than into the monolayers where the corresponding lipid had a longer chain length. This points to the importance of hydrophobic interactions for the incorporation. It was also observed that the incorporation was much faster into the lipid monolayer than into its own proteinous layer, being less "oil-like" than the lipid layer (Bos and Nylander, 1995). In addition, repulsive steric and electrostatic forces might contribute the lower rate of incorporation. Quinn and Dawson (Quinn and Dawson, 1969a) found that the threshold surface pressure, above which no penetration of cytochrome c took place in phosphatidylcholine monolayers, was considerably lower when DPPA was used instead of hydrogenated egg yolk phosphatidylcholine (e-PC).The latter lipid contained fatty acid with a longer chain length, about 60% C18 and 30% C16. Du et al. (Du et al., 1996) studied the influence of the alkyl chain length of glycolipids (dialkyl glycerylether-β-D-glucosides and dialkyl glycerylether-β-D-maltosides) on the interaction between lipid monolayers and glucose oxidase. The interaction, as shown by an increase in surface pressure, was found to increase with increasing lipid chain lengths for both types of lipids. These results suggest that the hydrophobic interaction is the predominant force. Furthermore it is interesting to note that the interactions were not so strong with the lipids having the more bulky head group, that is the dialkyl glycerylether-β-D-maltosides, although the Π-A isotherms for the corresponding dialkyl glycerylether-β-D-glucosides was similar. This illustrates that a bulky head group can sterically hamper the protein-lipid (hydrophobic) interaction.

Effect of Lipid Fluidity

The complete hydrogenation of e-PC was found not to affect the surface pressure threshold for penetration of cytochrome c compared to the native e-PC (Quinn and

Dawson, 1969a). However, the change in surface pressure due to the penetration of the protein versus initial surface pressure was less steep for the saturated one. A similar trend was observed for the e-PE samples (Quinn and Dawson, 1969b). The conclusion was that the limiting pressure for penetration to take place is likely to be determined by the work necessary for the penetration, that is $\int \Pi dA$, where an area of interface, A, has to be created for the protein to penetrate. Once the penetration is feasible the magnitude will depend on the space between the molecules and thus the degree of penetration is expected to be lower for the hydrogenated sample (Quinn and Dawson, 1969a). The surface pressure threshold below which penetration of cytochrome c into the anionic diphosphatidylglycerol (cardiolipin) monolayer took place was also found to decrease when the lipid was fully hydrogenated (Quinn and Dawson, 1969a). Ibdah and Phillips found the same trend in their study of the effect of lipid composition and packing on penetration of apolipoprotein A-I into lipid monolayers (Ibdah and Phillips, 1988). In the biological system this protein interacts with the phospholipid membrane of the serum high density lipoprotein (HDL) particles (see discussion in oil/aqueous interface section). Their results show that for this protein adsorption occurs to a larger extent on expanded monolayers than on condensed monolayers, that is, protein adsorption decreased in the order e-PC > egg sphingomyelin > DSPC. Furthermore it was found that protein adsorption generally decreased with increasing amount of cholesterol in the lipid monolayer. It was suggested this was due to the condensing effect of cholesterol.

5.4.1.3 Other Types of Surfactants

Blomqvist et al. (Blomqvist et al., 2004; Blomqvist et al., 2006) in vestigated the effect of the poly(ethylene oxide)-poly(propylene oxide) block copolymers F127 (PEO99-PPO65-PEO99), molecular weight 12500 g/mol, and P85 (PEO26-PPO39-PEO26), molecular weight 4600 g/mol on β-lactoglobulin foamability and foam stability. They found that the effect of the nonionic triblock copolymer on the interfacial rheology of beta-lactoglobulin layers is similar to that of low molecular weight surfactants (Blomqvist et al., 2004). However the protein foam stability was retained in the presence of the larger polymer F127, whereas P85 largely reduced the stability (Blomqvist et al., 2006). This shows that here the size of the amphiphilic polymer has a significant effect. The presence of F127 was found to increase thickness of the foam lamellae which in turn reflects the increased steric repulsion.

5.4.2 Influence of Protein and Protein Film Structure

The stability of the proteins largely affects the interaction with the emulsifier and the interface. Differences are observed between the random coil and globular proteins. The age of the surface layer of proteins that tend to aggregate can significantly decrease the penetration of the emulsifier in the surface layer.

Even though ionic surfactants may interact, more or less specifically with charged residues of proteins, especially so at low concentrations (see Sect. 5.3), no clear relation could be established regarding the influence of protein net charge on the interaction with ionic surfactants at high surfactant concentration (Wahlgren and Arnebrant, 1991; Wahlgren et al., 1993b; McGuire et al., 1995a). This might, of course, be related to the fact that in principle all proteins contain both negative and positive charges except at extreme pH. In an effort to determine key protein parameters for their interaction with surfactants, Wahlgren and coworkers studied the DTAB-induced removal of six adsorbed proteins: cytochrome c, bovine serum albumin, α-iactalbumin, β-lactoglobulin, lysozyme, and ovalbumin from silica and methylated silica surfaces (Wahlgren et al., 1993b). For silica surfaces, it was found that the removal of the proteins that were still adsorbed after rinsing with buffer, increased with decreasing molecular weight, adiabatic compressibility [a measure of conformational stability (Gekko and Hasegawa, 1986)] and increasing thermal denaturation temperature (Wahlgren et al., 1993b). In the case of hydrophobic (methylated silica) surfaces, differences between the proteins were smaller. However, increasing molecular weight and shell hydrophobicity of the protein seemed to reduce the degree of removal. It was also found that the removal did not relate to the degree of desorption of proteins upon rinsing with buffer, indicating that the mechanisms for the two processes are different. McGuire et al. (McGuire et al., 1995b) found that the removal of wild type and structural stability mutants of bacteriophage T4 lysozyme from hydrophobic and hydrophilic silica surfaces by a cationic detergent, decyltrimethylammonium bromide (DTAB), generally increased with the stability of the mutants.

The effect of the interfacial protein film age on the displacement of the protein from the surface of emulsion drops by nonionic water soluble surfactants [Tween 20 and octaethylene glycol *n*-dodecyl ether ($C_{12}E_8$)] showed that β-lactoglobulin is harder to replace the longer the residence time was (Chen and Dickinson, 1993; Chen et al., 1993). Similar results have been obtained for a range of other protein (Bohnert and Horbett, 1986; Rapoza and Horbett, 1990b). Apart from the possible conformational changes that occur during the adsorption process, which can hamper displacement, it has been reported that β-lactoglobulin might polymerize through disulphide exchange at the oil-water interface (Dickinson and Matsumura, 1991). Consequently, the displacement of β-casein, which is a flexible and unordered protein without sulfhydryl groups, did not depend on the age of the film. Furthermore it was observed that it was harder to replace β-lactoglobulin from a emulsion prepared close to the pI of the protein, than at neutral pH, whereas the replacement from emulsions prepared at pH 3 was easier and effect of the age of the protein film was observed. Mackie et al. also studied displacement of β-lactoglobulin and β-casein by Tween 20, but from the air-water interface (Mackie et al., 1999). They also found that β-casein was more easily displaced, i.e., β-lactoglobulin films breaks at higher surface pressures. Stress invoked by penetration of the surfactant was found to propagate homogenously through the β-casein film, which in turn resulted in growth of circular surfactant domains at the interface. β-Lactoglobulin, on the other hand was found to form elastic (gel-like) networks at the air-water interface and the penetration of the surfactant

therefore resulted in the growth of irregular (fractal) surfactant domains. Not surprisingly, Tween 20 preferentially displaced β-casein before β-lactoglobulin from a mixed β-casein/β-lactoglobulin film at the air-water interface (Mackie et al., 2001a).

5.4.3 Influence of Surface Properties

The surface properties affect the binding of the emulsifier as well as of the protein and has therefore large effect on the competitive adsorption.

The surface activity of the complex depends on the properties of the interface, as shown by Wilde et al. (Wilde and Clark, 1993) for liquid interfaces. They found that the complex between Tween 20 and β-lactoglobulin was more surface active at the oil-water interface than at the air-water interface, where the same surface activity as for the free (or pure) protein was observed. The complexes adsorbed at both type of interfaces were however displaced by Tween 20 at the same surfactant to protein ratio. Here, we need to emphasize the difference in nature between the two types of liquid interfaces, the liquid/air and the one between two condensed media, which explains the experimental observations. The oil/water interface allows hydrophobic residues to become dissolved in and interact favorably with the oil phase, which is not possible at the air/water interface. We have also previously discussed that the unfolding of protein induced by the action of surfactants or by the presence of an interface generally leads to exposure of hydrophobic residues, that is the unfolded protein can be substantially more "oil soluble" than the native one. This relates to the following section, dealing with molecular interactions, where it will be demonstrated, that changes in oil phase composition and hence solvent properties, also can lead to changes in the structure of the adsorbed protein film.

5.4.3.1 Solid-Liquid Interfaces (Dispersions and Macroscopic Surfaces)

Protein/surfactant interactions at solid-liquid interfaces have been studied with the aim of estimating the protein attachment strength to surfaces, for optimizing detergency processes, and for avoiding undesired adsorption in biomedical applications. The major part of the work has been carried out with the purpose of characterizing the protein binding to the surface rather than the protein/surfactant interaction and therefore concerned with the degree of removal, or elution, of adsorbed protein by surfactant. Even if the data mainly refer to solid surfaces, the basic principles are also valid at liquid interfaces such as those of the emulsion droplet. Since the process of surfactant interaction with proteins at interfaces is determined by the surfactant/protein, the surfactant/surface and protein/surface interactions, the following brief introduction is intended to provide a background on surfactant association and adsorption,

The adsorption and orientation of surfactants are dependent on the type of surface. There is a vast literature concerning the association of surfactants at solid/aqueous interfaces (Scamehorn et al., 1982; Manne et al., 1994; Zhmud and Tiberg, 2005;

Zhang and Somasundaran, 2006). The structure of the surface aggregates at the plateau has been debated, and surface micelles, finite bilayers, or infinite bilayers have been suggested for hydrophilic surfaces. It has been demonstrated that nonionic polyethylene glycol monoalkyl ethers (C_nE_m) adsorb as submonolayers or monolayers on hydrophobic surfaces, while they form surface micelles or bilayer type aggregates (depending on the type of surfactant) on hydrophilic surfaces (Tiberg, 1996) (Fig. 5.13). It is therefore natural to expect that the way in which surfactants interact with proteins should be influenced by the characteristics of the surface as well.

Wahlgren and Arnebrant (Wahlgren and Arnebrant, 1991) investigated the effect of the surface properties on the displacement of adsorbed β-lactoglobulin (negative net charge) and lysozyme (positive net charge) by the cationic surfactant cetyltrimethyl ammonium bromide (CTAB) and the anionic sodium dodecyl sulphate (SDS). They used hydrophobic (hydrophobised silica), negative (hydrophilic silica), neutral (chromium oxide) as well as positively charged (nickel oxide) surfaces and found four types of behavior for surfactant concentrations well above cmc:

1. *Surfactant binds to the protein and the complex desorbs on dilution.*

This was observed for SDS and β-lactoglobulin as well as lysozyme on negative silica surface and can be explained by simple electrostatic considerations. No adsorption from SDS/protein mixtures occurred.

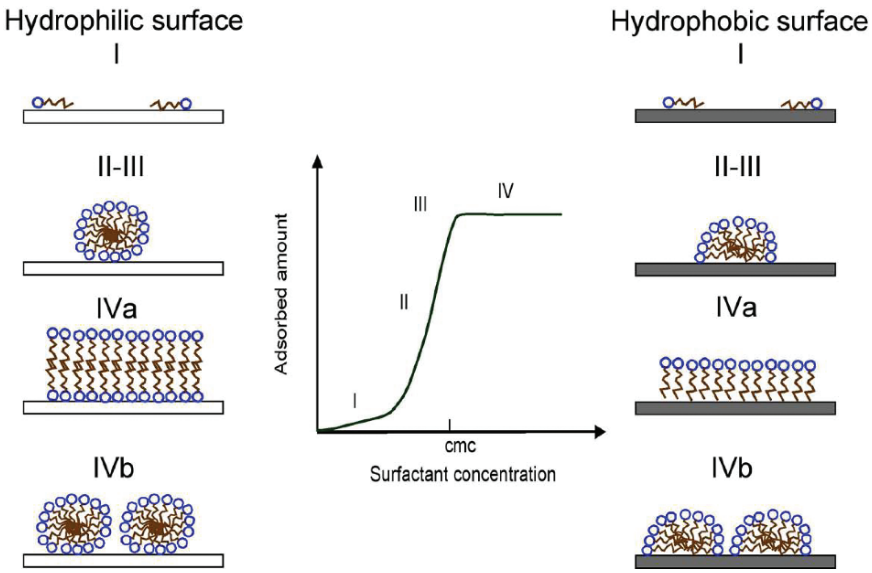

Fig. 5.13 An illustration of probable arrangements of adsorbed surfactant molecules at different degrees of surface coverage. Adsorption to hydrophilic surfaces (upper panels) and hydrophobic ones (lower panels). The illustrations are drawn to represent structures having minimal water contact with the hydrophobic parts of the molecules. The labels (I) to (IV) refer to structures that may exist in different regions of the isotherm. The figures should be considered as schematic and other structures, especially for ii to iii, have been suggested

2. *The surfactant replaces the protein at the interfaces*.

This requires that the surfactant interacts more strongly with the surface than the protein, as was observed for CTAB with negative silica and SDS and CTAB with the hydrophobic surface when the adsorbed layer consisted of β-lactoglobulin.

3. *The surfactant coadsorbs reversibly to the protein layer*.

The protein surface interaction is the stronger one and the surfactant is thus unable to solubilize the protein from the interface. This was observed for CTAB interacting with both proteins at the chromium oxide surface and SDS interacting with β-lactoglobulin at the nickel oxide surface.

4. *Partial removal of the protein*.

This can be explained as due to the presence of multiple binding sites for the protein, and can result from either mechanism 1 and 2.

Surface Charge

One can imagine several ways that emulsifiers can modulate the interaction of proteins with the surface depending on the charge of the surfactant, protein net charge and the surface charge. Here it is important to point out that ionic emulsifiers can affect the amount of protein on the surface by modifying the protein-surface interaction by changing the surface charge and/or protein charge as well as the interaction between adsorbed protein/emulsifier interaction.

Green et al. studied the interaction between sodium dodecyl sulfate (SDS) and preadsorbed lysozyme at the hydrophilic silicon oxide-water interface by neutron reflectivity measurements (Green et al., 2001). SDS binds cooperatively to the preadsorbed protein layer at intermediate surfactant concentrations, with no desorption of lysozyme from the interface. The protein was partly removed when the SDS concentration was increased to above 0.5 mM. While a surfactant concentration of 2 mM was required to completely remove both protein and surfactant from the interface. The surfactant–protein complex and the surface is then likely to both be negatively charged and the electrostatic interaction cause desorption.

Indirectly the neutron reflectivity study on the binding of SDS onto preadsorbed layers of bovine serum albumin (BSA) at the hydrophilic silicon oxide-water interface by Lu et al (Lu et al. 1998) confirm the "orogenic" displacement model (Mackie et al., 1999; Mackie et al., 2001a; Mackie et al., 2001b) discussed above. The specular neutron reflection is sensitive to the density profile normal to the interface, but does not give any lateral resolution. Their results suggest uniform layer distribution of SDS at low surfactant concentrations, while the distribution becomes unsymmetrical as the SDS concentration increases. The binding of SDS results in an expansion of the preadsorbed BSA layer from 35 Å in the absence of SDS to some 80 Å at 3×10^{-4} M SDS, which Lu et al. interpreted as a considerable structural deformation of the protein. They based this interpretation on the close agreement between the volume ratio of SDS to BSA in the mixed layer of 0.45, and the literature value for the binding of SDS onto denatured protein in the bulk reported by Tanner et al. (Tanner et al., 1982).

Investigations into the elutability of lysozyme and β-lactoglobulin from methylated silica (hydrophobic) and oxides of silicon, chromium, and nickel by SDS and cetyltrimethylammonium bromide (CTAB) showed no simple correlation between the fraction removed and the difference between the two oppositely charged surfactants. Instead, elutability of β-lactoglobulin and lysozyme decreased roughly in the order silica > chromium oxide > nickel oxide (Wahlgren and Arnebrant, 1991). In these cases the extent to which the protein is removed mainly reflects the binding mode of the protein to the surface.

Surface Hydrophobicity

Elwing et al. (Elwing et al., 1989; Elwing and Golander, 1990) studied the surfactant elutability of proteins adsorbed to a surface containing a gradient in hydrophobicity and found large differences in the amounts removed from the hydrophilic and hydrophobic ends. In the case of a nonionic surfactant (Tween 20), the elutability was largest at the midpoint of the gradient, which can be attributed to enhanced conformational changes of the adsorbed protein at the hydrophobic end, in combination with a lower efficiency of removal by nonionics at hydrophilic surfaces. At hydrophobic surfaces the removal is generally high (Elwing et al., 1989; Wannerberger et al., 1996). However, this may not be considered as evidence for weak binding of the proteins to the surface, but rather as an indication of the strong interaction between the surfactants and surface.

Horbett and coworkers (Bohnert and Horbett, 1986; Rapoza and Horbett, 1990b) studied the elutability of fibrinogen and albumin at different polymeric surfaces and found that the elutability and the change in elutability with time differed between surfaces. These differences could not, however, be correlated to surface energy in terms of their critical surface tension of wetting.

5.4.3.2 Liquid–Liquid Interfaces (Emulsions and Vesicles)

Most studies of protein–lipid interactions at the oil aqueous interface has been carried out using model emulsions. The purity of polar lipid and the way it is added (e.g., to the oil or the water phase) are bound to affect the interactions with proteins, which in turn affect the emulsion stability. Yamamoto and Araki (Yamamoto and Araki, 1997) studied this by comparing the interfacial behavior of β-lactoglobulin, in the presence of lecithin (PC) in the water or in the oil phase, with the stability of corresponding emulsions. In the presence of protein, crude lecithin was found to increase the stability of emulsion and lower the interfacial tension more effectively than a pure lecithin preparation. When crude lecithin was added to the oil phase the interfacial tension was found to decrease, and the emulsion stability increased as compared to when the lecithin was dispersed in the aqueous phase. One might speculate if these findings can be related to the presence of fatty acid and/or charged phospholipids in the crude lecithin. Aynié et al. studied the interaction between nitroxide homologues of fatty acids and milk proteins by following the mobility of

the nitroxide radicals using electron spin resonance (Aynié et al., 1992). At pH 7, the importance of the lipid protein interaction was not determined by the structure of the protein, but positively correlated with the number of positive charges on the protein. Thus, it was observed that the importance of the interaction in the emulsions decreased in the order α_{s1}-casein > β-lactoglobulin > β-casein, suggesting that the interaction was of electrostatic nature. The different proteins also affect the organization of lipid monolayer, where α_{s1}-casein in contrast to β-lactoglobulin and β-casein, induce an ordering of a monolayer of nitroxide fatty acids on the surface of an emulsion droplet (Aynié et al., 1992). This can probably be assigned to the stronger interaction of α_{s1}-casein with lipids compared to the other proteins.

Bylaite et al. applied ellipsometry to study the adsorption of the lipid from the oil and the protein from the aqueous phase at the oil–water interface (Bylaite et al., 2001). Independently of the used concentration, close to monolayer coverage of soy bean PC (sb-PC) was observed at the caraway oil-aqueous interface. On the other hand, at the olive oil – aqueous interface, the presence of only a small amount of sb-PC lead to an exponential increase of the layer thickness with time beyond monolayer coverage. This interesting observation was assigned to the formation of a multilamellar layer o sb-PC at the olive oil – aqueous interface, when sb-PC reached the solubility limit in the olive oil. The properties of the interfacial phase were found to depend strongly on whether phospholipid was added to the oil phase or to the aqueous phase as liposomal structures. In the latter case a monolayer formed, while if the phospholipid was supplied from the oil phase a lamellar phase appeared at the interface. The kinetics of the processes differs. Monolayer coverage from the liposomal dispersion is a rapid process, while the formation of the intermediate lamellar phase takes a much longer time. At very long equilibrium times (many days) the same equilibrium structure (lamellar phase at the interface) was formed. This observation agrees with presence of a third emulsifier phase at the O/W interface suggested by Friberg et al. (Friberg et al., 1969; Friberg, 1971). Westesen showed the existence of triple layers in lecithin stabilized vegetable oil emulsions using synchrotron X-ray scattering (Westesen and Wehler, 1993), but for their system they found that not more than a monolayer is needed for stable emulsions. The addition of β-lactoglobulin has also little effect on the formation and the formed DOPC layer when the DOPC is dispersed in the oil phase.

Bylaite et al. also studied the stability and droplet size of β-lactoglobulin and lecithin (phosphatidylcholine from soybean, sb-PC) stabilized emulsions of caraway essential oil as well as the amount of protein on the emulsion droplets (Bylaite et al., 2001). It should be noted that sb-PC was dispersed in the oil phase. Some of their data are given in Fig. 5.14, where the amount of β-lactoglobulin adsorbed on the oil aqueous interface is shown versus amount added s-PC. These data show that sb-PC is likely to replace some of the protein at the oil – aqueous interface, although it is unable to completely replace the protein. The maximum reduction in the amount of β-lactoglobulin adsorbed is by a factor of 3 for the caraway oil. These findings are in agreement with other studies, where lecithin was found to be less efficient in displacing milk proteins from the oil/water interface compared to other surfactants (Courthaudon et al., 1991; Dickinson and Iveson, 1993).

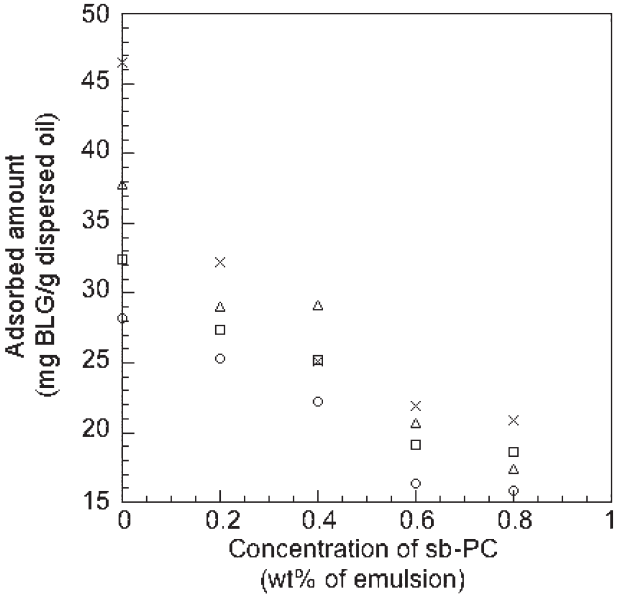

Fig. 5.14 Adsorbed amount of protein at the caraway essential oil –water (Δ, ×) and olive oil –water (O, ❑) interfaces in emulsions stabilized by 1 (Δ, O) and 2 (×, ❑) wt.% β-lactoglobulin and variable amount of soybean-PC. Emulsions were prepared from 15 wt.% oil in a 60-mM phosphate buffer of pH 6.7. Data adapted from Bylaite et al. (2001), where also the experimental details are given

The displacement of caseinate from the interface of emulsion droplets by monoglycerides, monooleoylglycerol and monostearoylglycerol, dissolved in the oil phase was found to correlate with the adsorption of the monoglycerides at the oil–water interface (Heertje et al., 1990). The amount of monooleoylglycerol increased gradually with concentration and reached a plateau when approaching an oil phase concentration of 1 wt%. Under these conditions all of the caseinate was displaced from the interface. The saturated lipid, monostearoylglycerol, was much more efficient in displacing the protein. Already, at a concentration in the oil phase of between 0.2 and 0.3 wt% the adsorbed amount of monostearoylglycerol increased sharply and reached much higher surface concentrations than monooleoylglycerol. At 0.3 wt% all of the caseinate was removed from the interface.

Protein Interactions with Lipid Vesicles

The mechanisms that determine the stability, size and shape of vesicles are complex and widely discussed (for reviews see for instance Helfrich, 1989; Lasic, 1993; Komura, 1996; Lasic et al., 2001). The spherical shape is generally the most stable shape for equal distribution of molecules between the two monolayers constituting the bilayer (Lasic, 1993). These spherical vesicles can be large multilamellar vesicles

(MLV), and large (LUV) and small (SUV) unilamellar vesicles (Lasic, 1993). The bending of the lipid bilayer to form a vesicle imposes a strain on a symmetric bilayer as the inner monolayer has a negative curvature, while the outer has a positive curvature. The magnitude of this curvature energy can be difficult to estimate, but it is thought to be significant enough to in many cases make the vesicles inherently unstable and energy has to be added to form them (Lasic, 1993; Komura, 1996; Lasic et al., 2001). The result of the tension can be nonspherical vesicles (Seifert et al., 1991). A mixture of phospholipids, which corresponds to the composition in the milk fat globule membrane, gives both spherical vesicles and tubular structures (Waninge et al., 2003). In particular compositions (e.g., 80% DOPE, 12% DOPC and 8% sphingomyelin) that at high lipid content give liquid crystalline phases at the boundary of lamellar to reversed hexagonal phase tend to give microtubular structures at high water content rather than vesicles. A larger proportion of multilamellar vesicles were observed in buffer and divalent salts than in pure water. A small increase in the interlayer spacing of the multilamellar vesicle was observed in the presence of β-lactoglobulin and β-casein.

Driving Force for the Protein-Vesicle Interaction

The driving mechanism for the interaction of proteins with the lipid bilayer of the vesicles are basically as for the interaction a lipid monolayer at the air-aqueous interface. In parallel to the Quinn and Dawson study discussed above (Quinn and Dawson, 1969b; Quinn and Dawson, 1969a), Rytömaa et al. (Rytömaa et al., 1992) found a strong electrostatic contribution when cytochrome c binds to cardiolipin-phosphatidylcholine liposomes. This interaction did not take place if the negatively charged lipid cardiolipin was absent in the membrane. Furthermore, the protein was dissociated from the vesicle in the presence of 2-mM $MgCl_2$ and 80-mM NaCl at pH 7. The apparent affinity of cytochrome c to the vesicles also increased when the pH was dropped to 4. The interaction was found to be completely reversible for pH changes, that is, if the pH was increased to 7, the protein could be dissociated from the vesicle by adding salt.

Price et al. studied the adsorption of fibrinogen to neutral liposomes, composed mainly of phosphatidylcholine (PC) and cholesterol and negative liposomes, composed mainly of phosphatidic acid (PA) and cholesterol, as well as to the corresponding liposomes in which a PEG-modified phosphatidylethanolamine had been introduced (Price et al., 2001). They found that negatively charged liposomes adsorbed more fibrinogen than the corresponding neutral liposomes. PEG modification was found to have no effect on neutral liposomes in terms of fibrinogen adsorption. However, PEG modification, which sterically stabilizes the liposomes, markedly reduced the adsorption to the negative liposomes.

Brooksbank et al. conducted an extensive study on the interaction of β-casein, κ-casein, α_{s1}-casein, and β-lactoglobulin with negatively charged egg yolk phosphatidylglycerol (PG) and zwitterionic egg yolk phosphatidylcholine (PC) vesicle using photon correlation spectroscopy (Brooksbank et al., 1993). Their data on the

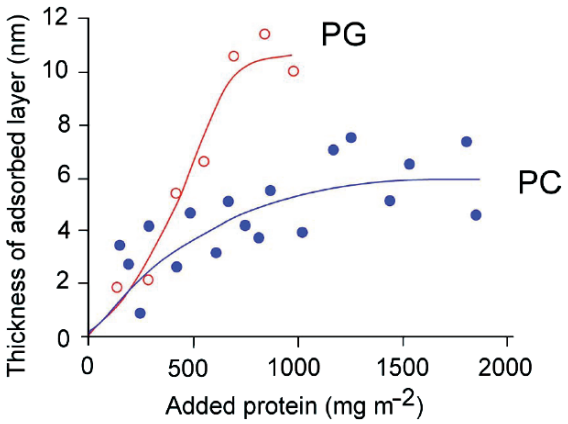

Fig. 5.15 Thickness of adsorbed layer of β-casein on negatively charged egg yolk phosphatidylglycerol (PG) and zwitterionic egg yolk phosphatidylcholine (PC) vesicle as a function added protein expressed as μg of protein per square meter of available liposome surface. The liposomes were dispersed in 160 mM and the pH was about 6.2. The data are taken from a photon correlation spectroscopy study by Brooksbank et al. (1993), where further experimental details are given

adsorption of β-casein are shown in Fig. 5.15. All of the studied proteins were found to give a thicker layer on the negatively charged vesicles, although they all carried a negative net charge under the conditions used (160-mM sodium chloride at pH 6.2). Brooksbank et al. (Brooksbank et al., 1993) suggested that binding to the vesicle surface takes place mainly through hydrophobic interactions and the differences in thickness of the adsorbed layers on the two types of vesicles were explained in terms of the protein charge distribution. For instance the hydrophilic, N-terminal, part of β-casein has a net charge of −12, whereas the remainder of the molecule carries almost no net charge. Thus, on the negatively charged vesicle surface, the molecules adopt a more extended configuration as the N-terminal part is likely to be pushed away from the surface by means of electrostatic repulsion. This explains the thicker layers on this surface as shown in Fig. 5.15. A similar reasoning can be applied for κ-casein. The apparently very thick adsorbed layer of α_{s1}-casein was explained by bridging flocculation of the vesicles mediated by the protein. The middle section of α_{s1}-casein carries a negative net charge, while the two ends have no net charge. One of the uncharged ends pertrudes into the vesicle bilayer and the middle section is repelled from the vesicle surface, leaving the other uncharged end of the peptide chain free to interact with another vesicle. The charge distribution on β-lactoglobulin is more even and the interpretation of the results was not as straightforward.

As discussed by Kinnunen the introduction of a H_{II} forming double chain lipid (a lipid with packing parameter > 1, see Fig. 5.2) in a lamellar membrane can impose a considerable stress on the membrane (Kinnunen, 1996). This frustrated membrane is said to be in the L_{ε} state according to the Kinnunen terminology (Kinnunen, 1996).

Free energy can be gained by allowing some of the lipids in the frustrated membrane to adopt the so-called extended or splayed chain conformation, where one of the acyl chains extends out from the bilayer, while the other chain remains in the membrane. Such an extended chain can also become accommodated within a proper (hydrophobic) cavity of a protein interacting with the membrane (Kinnunen 1996). This is an interesting alternative explanation for the hydrophobic interaction between peripheral proteins and membranes that has been discussed in this review. The splayed chain conformation has also been suggested to be one mechanism for membrane fusion (Kinnunen and Halopainen, 2000). This and other implications of the splayed chain confirmation has been discussed by Corkery (Corkery, 2002).

Influence of the Protein Structure on the Vesicle Interaction

Kim and Kim studied the interaction between α-lactalbumin and phosphatidylserine/phosphatidylethanolamine vesicles (1:1 molar ratio) versus pH (Kim and Kim, 1986). They found that the interaction, which almost did not exist at neutral pH, increased with decreasing pH (Fig. 5.16). What is interesting to note (Fig. 5.16), is

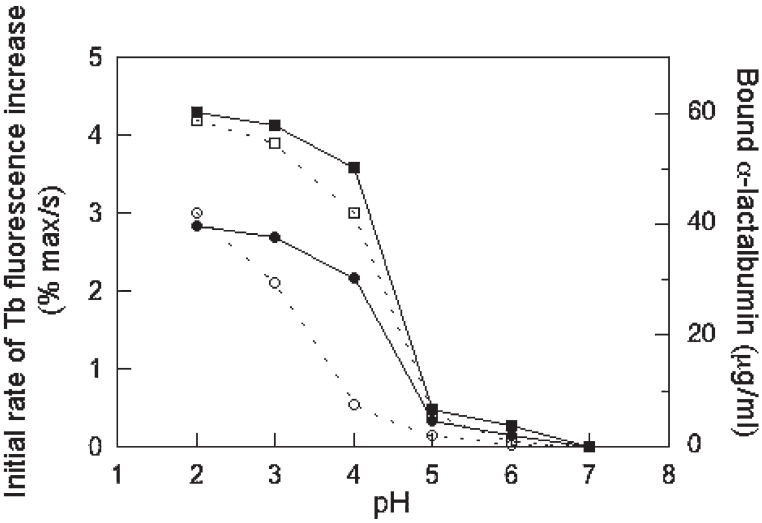

Fig. 5.16 The initial rate of Tb fluorescence increase (--- ❍ - - -, - - - ❑ - - -) upon α-lactalbumin induced fusion of phophatidylserine/phosphatidylcholine (1:1 molar ratio) vesicles is shown as a function of pH. The pH-dependent binding of α-lactalbumin is shown as the amount of protein bound per ml vesicle suspension (●, ■), which contained 1-mM lipid molecules (determined from the phosphorous content) per ml suspension. The results for initial protein concentrations of 50 (❍, ●) and 100 (❑, ■) μg/ml are presented. As the curves for the fusion process represents kinetic data and the binding studies represent equilibrium data when the fusion process is over, only qualitative comparison is possible. Data adapted from Kim and Kim (1986), where also the experimental details are given

that vesicle fusion, as estimated from increase of the initial rate of Tb fluorescence increase, correlates with the binding of the protein to the vesicles. The binding was suggested to be due to hydrophobic interaction via protein segments penetrating into the lipid bilayer as it was impossible to dissociate it by increasing the pH. This was further confirmed by using proteolytic enzymes, which were found to cut off both ends of the polypeptide chain leaving only the segment that penetrate into the bilayer. This penetrating protein loop was also believed to induce fusion of the vesicles.

The importance of the protein conformation on the interaction with vesicles was also shown in the study of Brown et al. (Brown et al., 1983). They found no interaction between native β-lactoglobulin and DPPC vesicles, but β-lactoglobulin, modified by exposing it to a 2:1 mixture of chloroform and methanol, did interact with the vesicles. Moreover, the lipid–protein complex formed had an α-helix content of at least 25–30% larger than for the native protein. The interaction was found to lead to aggregation of the vesicles at pH 7.2, while no aggregates were observed at 3.7. This was explained by the larger net charge at pH 3.7 (+20) compared to pH 7.2 (−10). These results imply that protein modification, either during processing or by special treatment, can increase the helix content, which in turn can be boosted by lipid interaction. The lipid–protein complexes formed in such a way have been suggested as a way to improve the emulsification processes (Brown, 1984; de Wit, 1989).

Lateral Phase Separation in Vesicle Bilayers

Raudino and Castelli reported that the presence of lysozyme could induced lateral phase separation in vesicle bilayers composed of a mixture of phosphatidic acid and phosphatidylcholine (Raudino and Castelli, 1992). Their differential scanning calorimetry study of the lipid chain melting transition showed good mixing in absence of the protein and the single peak was shifted towards higher temperatures as the phosphatidic acid content increased. In the presence of lysozyme, however, the chain melting transition peak was split into two peaks, indicating a lateral phase separation. In addition they found that temperature of protein unfolding increased with the fraction of phosphatidic acid, suggesting a stabilization of the protein due to the interaction with phosphatidic acid.

It is important to bear in mind that microheterogeneity of the bilayer does not only occur for mixtures of different lipids, but also close to the gel-to-fluid phase transition of the lipid. Hønger et al, studied the relation between phospholipase A_2 catalyzed hydrolysis of one component phosphatidylcholine vesicles and the microheterogeneity of the lipid bilayer (Hønger et al., 1996). They varied the microheterogeneity by changing the temperature in the vicinity of the gel-to-fluid phase transition as well as using lipid chain lengths between C14 to C18 and found a strong correlation between the maximal lipase–lipid interaction and the maxima in interfacial area between gel and fluid domains.

5.4.3.3 Liquid-Air Interfaces (Foams)

Emulsifiers with High Aqueous Solubility

Adsorption of Emulsifier–Protein Complexes

Tween 20 and β-lactoglobulin are known to interact in solution to form a 1:1 complex characterized by a $K_d = 4.6\,\mu M$, which has an increased hydrodynamic radius of 5.7 nm compared to 3.5 nm for β-lactoglobulin alone (Clark et al., 1991a). Detailed measurements of the properties of foam films formed from a constant concentration of 0.2 mg/mL mixed native and fluorescein-labeled β-lactoglobulin as a function of increasing Tween 20 concentration (Wilde and Clark, 1993; Clark, 1995) have been reported. This study revealed that between molar ratios *(R)* of Tween 20 to β-lactoglobulin of 0.2 to 0.9, there was a progressive increase in the thickness of the foam films and a corresponding decrease in the amount of adsorbed protein to an intermediate level of approximately 50% of that which was originally adsorbed. These changes occurred prior to the onset of surface diffusion of the labeled protein as determined by the FRAP technique at $R = 0.9$ (Coke et al., 1990). One persuasive interpretation of the data is that coadsorption or trapping of the Tween 20/β-lactoglobulin complex in the adsorbed multilayers could account for adsorbed-layer thickening (Clark et al., 1994a), since the complex is known to have an increased hydrodynamic radius (Clark et al., 1991a). However, further studies have showed that the increase in thickness was mainly due to the displacement of the protein by the surfactant. AFM studies showed that surfactant domains were formed which expanded and compressed the protein rich matrix (Mackie et al., 1999) increasing its thickness prior to complete displacement of the protein. This phenomena was observed in all protein surfactant systems despite the absence of specific protein–surfactant interactions (Mackie et al., 2001b; Mackie and Wilde, 2005). Comparing nonionic and ionic surfactants showed that the headgroup nature had specific impacts on the structure of the interfacial film. Nonionic surfactants generally formed domains in the protein matrix, which expanded as more surfactant was added. However, ionic surfactants (both anionic and cationic) both formed a greater number of smaller domains than nonionic surfactant (Gunning et al., 2004) and displaced the protein via the minimal expansion of a larger number of domains. Computer simulations also showed similar surface structures when the interaction potentials between the proteins and surfactants were varied (Wijmans and Dickinson, 1999; Pugnaloni et al., 2004). This suggested that nonionic surfactants in general had a net repulsive interaction with adsorbed proteins, probably due to steric repulsion, whereas ionic surfactants had a relatively more attractive interaction with adsorbed proteins. This is probably due to the fact that although the protein has a net charge, they are polyelectrolytes with both negative and positive charges, thus, some parts of the protein will be attracted to an ionic surfactant, irrespective of its charge.

Further evidence supporting direct adsorption of the complex formed between β-lactoglobulin and Tween 20 comes from dynamic surface tension (γ_{dyn}) measurements performed using the overflowing cylinder apparatus (Clark et al., 1993). Inclusion of β-lactoglobulin (0.4 mg/mL) in the initial solutions caused only a small reduction in

the measured γ_{dyn} to 71 mN/m. This remained unaltered in the presence of Tween 20 up to a concentration of 15 μM. Above this concentration a small but significant further reduction in γ_{dyn} was observed. The effect resulted in a small inflection in the γ_{dyn} curve in the region corresponding to 15 to 40 μM Tween 20. At higher Tween 20 concentrations, the curve for the mixed system followed that of Tween 20 alone. The inflection in the γ_{dyn} isotherm observed for the mixed system at concentrations of Tween 20 greater than 10 μM could not be due to adsorption of Tween 20 alone since under the prevailing conditions, the concentration of free Tween 20 was reduced by its association with β-lactoglobulin. Using Equation (5.5) it can be shown that the Tween 20/β-lactoglobulin complex is the dominant component in solution in the Tween 20 concentration range of 15 to *35* μM (Clark et al., 1993).

Direct adsorption of complex at the air/water interface also appears to have importance in functional properties of certain lipid-binding proteins from wheat called "puroindolines" (Wilde et al., 1993; Dubreil et al., 1997; Biswas and Marion, 2006). These proteins show unusual behavior in the presence of lipids that they bind, in that their foaming properties are generally unaltered and in some cases enhanced. A systematic study of the influence of interaction with lysophosphatidyl cholines (LPC) of different acyl chain lengths and has produced persuasive evidence of the importance of the complex on foaming activity (Wilde et al., 1993). First, two isoforms of the protein were investigated, puroindoline-*a* and -*b* (the *b* form has also been referred to as "friabilin"). Puroindoline-*b* has a significantly increased K_d for LPC compared to puroindoline-*a* (i.e., 20-fold weaker binding) and the enhancement of foaming properties is correspondingly reduced in the *b* form. Further studies of the binding of LPC to the *a* form revealed that the binding became tighter with increasing acyl chain length, and higher concentrations of the short-chain-length LPC are needed to achieve optimal foam stability enhancement (Husband et al., 1995). Lauryl-LPC showed no interaction with the puroindoline-*a* until the levels present exceeded the critical micelle concentration of 400 μM. This indicates a cooperative binding since it takes place in this concentration range, and any of the suggested structures for the protein/surfactant complexes, e.g., the pearl and necklace structure (Fig. 5.6), could be applicable. It seems increasingly likely that the functional properties of the puroindolines are linked to a role in the transport and spreading of lipid at the air/water interface.

When comparing the data for the interaction between SDS and ovalbumin and the corresponding data for BSA we clearly observe the different mode of interaction (Fig. 5.17b). The gradual decrease in surface tension with increasing surfactant concentration observed for ovalbumin and SDS mixtures can be explained by more efficient packing at the interface as discussed below. In addition, it has been argued that the attractive electrostatic interaction between surfactant and protein might increase the hydrophobicity and hence the surface activity of the protein. The specific binding of SDS to BSA does not affect the surface tension until the concentration corresponding to saturation of the high affinity binding sites is reached, that is 9–10 mole SDS per mole protein (Makino, 1979), where a sharp decrease in surface tension is observed. This arises probably from an increase in the free monomer concentration of SDS. The second plateau, indicating constant surfactant monomer concentration,

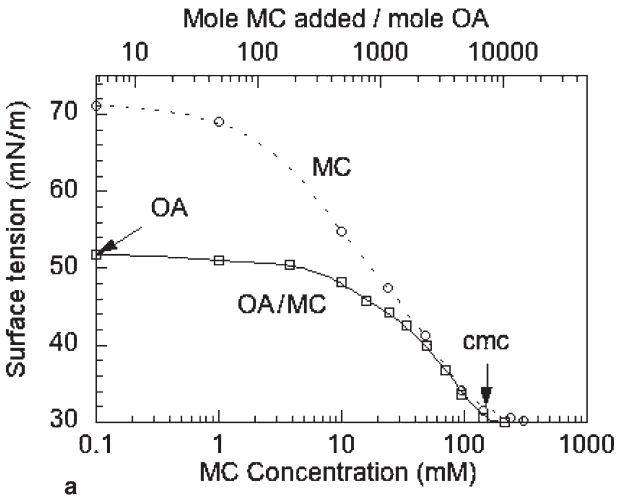

Fig. 5.17a Surface tension isotherms of 21 μM ovalbumin (OA) (❑) in the presence of the nonionic monocaproin (MC) in water adjusted to pH 5.6, where the surface tension of the pure protein is marked with an arrow on the ordinate. Surface tension of pure MC is also shown (❍) and the cmc is marked with an arrow on the abscissa. The surface tension measurements were performed according to the drop-volume method as a function of time. The surface tension value after 2000 s has been used for the isotherms. Further details are given elsewhere (Ericsson and Hegg 1985)

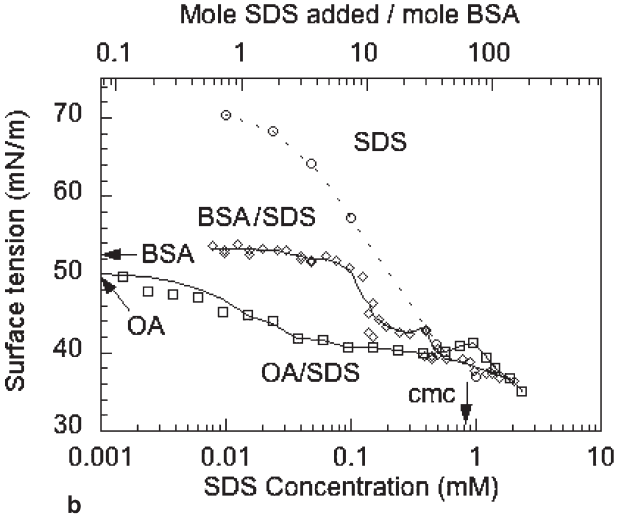

Fig. 5.17b Surface tension isotherms of 21-μM ovalbumin (OA) (❒) and 13-μM bovine serum albumin (BSA) (◊) in the presence of the anionic sodium dodecylsulphate (SDS) in 0.05-M phosphate buffer, pH 5.6. The surface tension of the pure proteins are marked with arrows on the ordinate. Surface tension of pure SDS is also shown (❍) and the cmc is marked with an arrow on the abscissa. Other conditions are the same as given under Fig. 5.17a

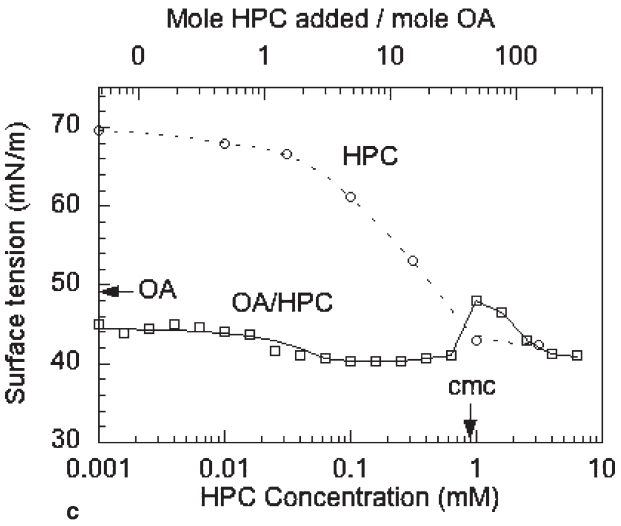

Fig. 5.17c Surface tension isotherms of 21-μM ovalbumin (OA) (❑) in the presence of the cationic hexadecylpyridinium chloride (HPC) in water adjusted to pH 4.0. The surface tension of the pure protein is marked with an arrow on the ordinate. Surface tension of pure HPC is also shown (❍) and the cmc is marked with an arrow on the abscissa. Other conditions are the same as given in Fig. 5.17a

which is observed at increased surfactant concentration is likely to be connected with saturation of the cooperative binding sites. As surfactant concentration further increases the surface tension isotherms for the two protein surfactant mixtures coincide. The second plateau observed in surface tension isotherms for ovalbumin and HPC mixtures just below cmc of HPC (Fig. 5.17c), can be related to the electrostatic interaction between HPC and globular proteins that has been observed below cmc in bulk solution (Ericsson et al., 1987a). It is noteworthy that the surface tension is slightly lower than for pure HPC, suggesting that the complex is more surface active. Green et al. used specular neutron reflection and surface tension measurements to study the adsorption of lysozyme and SDS at the air–water interface (Green et al., 2000). Their results show that the lysozyme-SDS complexes are much more surface active than the unbound species as the surface excesses for both lysozyme and SDS increases and surface tension decreases upon addition of SDS (region A). Interestingly the molar ratio of SDS to lysozyme was found to remain constant at about 7, although the total surface excesses increase with SDS concentration up to a surfactant concentration of 2.5×10^{-4} M. This indicates that the complex that adsorbed on the interface had a rather well-defined stoichiometric composition. Further increase in SDS concentration beyond 2.5×10^{-4} M lead to a sharp decrease in the total surface excess, while the molar ratio of SDS to lysozyme increased. Eventually, as more SDS was added, the mixed protein/surfactant layer was replaced by a pure SDS monolayer. The zwitterionic surfactant LPC was found to enhance the foaming

properties of β-lactoglobulin (Sarker et al., 1995). An enhanced adsorption of this complex, and an increase in the elastic properties of the mixed interface were also found, which could be linked with enhanced electrostatic interactions between the adsorbed protein and surfactant (Gunning et al., 2004).

Decreased Surface Activity of the Emulsifier–Protein Complex

The maxima in the surface tension isotherm at HPC concentrations between 0.8 and 2.5 mM probably reflects an increased HPC-ovalbumin interaction in bulk solution (Fig. 5.17b). The formed highly charged complex is less surface active and an increase in surface tension is thus observed. The surface tension maximum has been found to depend on ovalbumin concentration, and is shifted towards higher HPC concentration at increased ovalbumin concentration (corresponds to 30 mole HPC per mole ovalbumin, independent on protein concentration) (Ericsson, 1986). The adsorption from mixtures of human serumalbumin (HSA), and nonionic surfactant, decyl-dimethyl-phosphine-oxide (C_{10}DMPO) at the air–water interface was reported by Miller et al. (Miller et al., 2000b). They reported an anomalous surface tension increase for the mixtures at low surfactant concentrations to values higher than for the protein at the same concentration without the surfactant. Thus it seemed that surfactant protein complex was less surface active. The likely explanation is that the nonionic surfactant associate with HSA via hydrophobic interaction and thus makes the protein more hydrophilic and hence less surface active. Miller et al. also observed that the concentration range, where the coverage of protein and surfactant are comparable in the mixed surface layer was quite narrow (Miller et al., 2000b).

The precipitation of protein in the bulk solution due to neutralization by added surfactant can also cause a decrease in surface concentration due to loss of surface active material. Garcia Dominguez et al. (Garcia Dominguez et al., 1981) have shown that the surface tension reduction of lysozyme and insulin at pH 3.5 (i.e., below pI) decreased when an anionic surfactant (SDS) was added, due to precipitation of the protein.

The Lateral Electrostatic Interactions Can Control the Layer Composition

A synergistic effect on surface tension is seen for mixtures of proteins with both the anionic and cationic surfactant (Fig. 5.17b and c). For ovalbumin and SDS mixtures (Fig. 5.7b), a gradual decrease of the surface tension with increasing surfactant concentration is observed. This might be assigned to the more efficient packing in the formed mixed surfactant /protein layer compared to the one formed by the individual components at this concentration (Ericsson and Hegg, 1985). Even at the lowest concentration of cationic surfactant (0.05 mole HPC per mole ovalbumin), where the pure surfactant has the same surface tension as water, a decrease in surface tension for the protein surfactant mixture, compared to pure ovalbumin, is observed (Fig. 5.17c). It is unlikely that any bulk interaction will affect the interfacial behavior at this low HPC to ovalbumin ratio. Therefore the lowering in the surface tension probably arises from molecular interactions in the adsorbed surface film, giving a more

condensed surface layer. Buckingham et al. (Buckingham et al., 1978) found strong synergistic lowering of the surface tension of a mixed solution of SDS and poly-L-lysine at conditions at which no precipitation, micelle or complex formation take place in the bulk solution. Similar behavior was observed in mixtures of low molecular weight surfactants of opposite charges (Lucassen-Reynders et al., 1981). This effect has been assigned to the reduction of electrostatic repulsion within the layer.

5.4.3.4 Emulsifiers with Low Aqueous Solubility

The large number of studies using lipid monolayers at the air/aqueous interface and spread or adsorbed proteins have given us the basic knowledge of the interaction between proteins and polar lipids with low aqueous solubility.

Monolayer Stability

One might expect that monolayer made up of lipids with very low aqueous solubility would be stable. However, this is far from general. Metastablility of monolayers, can be caused by processes such as rearrangement within the layer, dissolution into the sub-phase and transformation to a three dimensional phase, which can occur at pressures above the equilibrium spreading pressure (Vollhardt, 1993; Vollhardt et al., 1996). Furthermore, the stability of the monolayers can be affected by the spreading solvent and the techniques used for spreading the lipid (Gericke et al., 1993; Carlsson et al., 1995) The stability of the monolayer can also be considerably changed by the ion composition of the aqueous sub-phase. For instance the stability of an arachidic (n-eicosanoic, C20:0) acid monolayer was found to increase in the order

$$H^+ < Li^+ < Na^+ < Ca^{2+} < Mg^{2+}$$ (Vollhardt, 1993).

There are several examples of proteins that are thought to have the role to stabilize a lipid mono- or bilayer. One such example is the milk fat globule membrane that has been suggested to consist of the monolayer of polar lipids, which covers the fat globule surface, and an outer lipid based bilayer (Danthine et al., 2000; Mather, 2000). The milk fat globule membrane is expected to be inhomogeneous with significant amount of proteins in the membrane. An aqueous layer containing different proteins, like xanthine oxidase, is present between the monolayer and bilayer. One of the roles that have been assigned to xantinoxidase is to stabilize the milk fat globule membrane (Mather, 2000). Interestingly, Kristensen et al, found that the presence of a xanthine oxidase can increase the stability of a monolayer composed of sphingomyelin from the milk fat globule membrane (Kristensen et al., 1996). They investigated the interaction between one of the major proteins, xanthine oxidase, and the major lipids, sphingomyelin and phosphatidylcholine, in the milk fat globule membrane at the air / aqueous interface by using the monolayer technique. Both lipids have a similar

phopshorylcholine headgroup, which is zwitterionic in the neutral pH range, although the belt regions linking the phopshorylcholine group with the acyl chains are different. The Π-A isotherms of sphingomyelin and phosphatidylcholine are shown in Fig. 5.18a and b, respectively. The isotherms for sphingomyelin monolayers spread

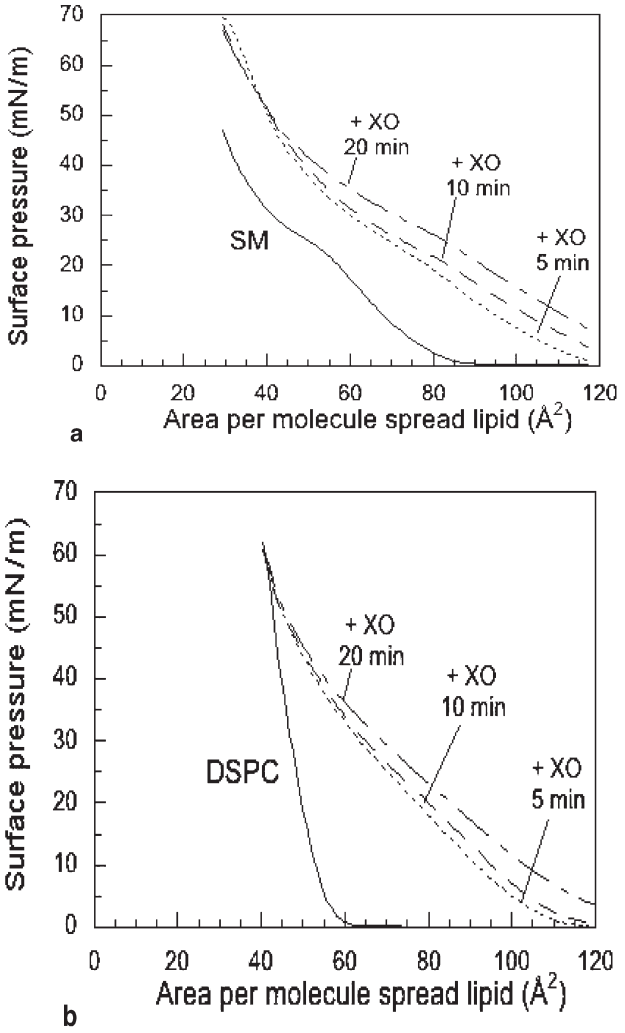

Fig. 5.18 Dynamic surface pressure (Π) as a function of the molecular area of the spread amount lipid for compression of (a) sphingomyelin and (b) distearoylphosphatidylcholine (DSPC) monolayers on a phosphate buffered subphase (40-mM phosphate containing 0.1-M sodium chloride, pH = 7.4) with or without xanthine oxidase (5 mg/ml). The isotherms recorded for the lipid spread on pure buffer (-) and at 5 (- - - - -), 10 (— — — —), 20 (— - — -) min elapsed between spreading on xanthine oxidase solution and compression. The lipid (25 μg) was spread from a chloroform/methanol (2:1, v/v) solution on a maximum area of 50 × 450 mm^2 and a compression speed of 12.5 mm/min was used. Data adapted from Kristensen et al. (1996), where also the experimental details are given

on pure buffer and a xanthine oxidase solution are shown. The slope of isotherm and the area of the compressed monolayer for pure sphingomyelin (Fig. 5.18a) are smaller than expected for these types of lipids. In addition, the large hysteresis and the dependence on the compression speed, not observed for distearoylphosphatidylcholine, confirms that the sphingomyelin monolayer is metastable. The difference in stability of monolayers formed by two different lipids can probably be related to the different conformation of choline groups in the two types of lipids, where intra molecular hydrogen bonding is possible between the phosphate group and the amide and hydroxyl groups in the belt region of sphingomyelin (Siminovitch and Jeffrey, 1981). An increase in m; at maximum compression of the sphingomyelin monolayer, which reflects an increase in the monolayer stability, was observed in the presence of sphingomyelin. Furthermore, the area per sphingomyelin molecule increases in the presence of xanthine oxidase even at high Π-values. This is in contrast to the results from the parallel study of the phosphatidylcholine monolayers with and without xanthin oxidase, where the interacting protein could be completely squeezed out from the lipid monolayer at high enough surface pressures without affecting the collapse pressure. This indicates that interaction between xanthine oxidase and sphingomyelin is much stronger than that between the protein and phosphatidylcholine.

Structure of the Interfacial Film

Even from the study of the penetration of protein versus surface pressure it is also possible get some hints about the structure of the mixed layer. Cornell et al. (Cornell, 1982; Cornell and Patterson, 1989; Cornell et al 1990) observed penetration of β-lactoglobulin, α-lactalbumin or BSA into mixed monolayers of POPC and POPG at such high surface pressure that it is unlikely that the proteins could penetrate into a protein layer. Thus, they concluded that the formation of pure protein patches is unlikely and that portions of the protein are suggested to be intercalated into the lipid monolayer. Bos and Nylander made similar observation for the interaction between β-lactoglobulin and DSPC and DSPA monolayers (Bos and Nylander, 1995).

Fluorescence microscopy and Brewster angle microscopy (BAM) can be used to in situ image the structure of the film at the air/aqueous interface, although the lateral resolution is limited by the resolution of the optical microscope. Fluorescence microscopy together with surface film balance technique was used to by Heckl et al. to study the structure of mixed phospholipid-cytochrome c and b films (Heckl et al., 1987). They found that proteins mainly were located in the fluid membrane phase, which coexisted with solid lipid domains without protein. The penetration into the lipid monolayer was reduced with increasing pressure. Cytochrome c (positively charged) was found to interact with dimyristoylphosphatidic acid (DMPA) monolayers but not with dipalmitoylphosphatidylcholine (DPPC) layers, showing the electrostatic nature of the interaction. Schönhoff et al. concluded from their study of the incorporation of membrane proteins into DPPA/DOPA monolayers that incorporation mainly takes place in the fluid phases of the matrix (Schönhoff et al., 1992). Zhao et al. used BAM to image the kinetics of β-lactoglobulin penetration

into DPPC monolayers at the air-aqueous interface from a 500-nM solution in 10-mM phosphate buffer, pH 7 (Zhao et al., 2000). For instance at an initial surface pressure of 7.8 mN/m, it took 0.17 min until domains, with similar morphology as those appearing during the compression of a pure DPPC monolayer, appeared. These domains were found to consist only of the lipid as confirmed by grazing incidence X-ray diffraction and β-lactoglobulin penetration was found to occur without any specific interaction with DPPC. β-Lactoglobulin was not able to penetrate into a condensed DPPC monolayer, that is, above surface pressure of about 20 mN/m.

The lateral organization in mixed protein–lipid films at air-aqueous interface can be studied by spectroscopic techniques and high resolution imaging techniques such as electron microscopy and atomic force microscopy (AFM) after transferring the films to a solid support. Using electron microscopy Cornell and Carroll found that only lipids with the chains in liquid state, e-PA, dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine, formed homogenous films with β-lactoglobulin, whereas DPPA and DSPC formed heterogeneous layers (Cornell and Caroll, 1985). AFM as powerful technique to study the lateral organization in mixed films of proteins and soluble surfactant s have already been demonstrated with the development of the "orogenic" displacement model (Mackie et al., 1999; Mackie et al., 2001a; Mackie et al. 2001b). Diederich et al. studied the interaction between bacterial surface layer proteins (S-layer proteins) and phosphatidylethanolamine (DMPE and DPPE) monolayers using dual label fluorescence microscopy, FTIR spectroscopy, and electron microscopy (Diederich et al., 1996). When the monolayer is in the two-phase region, with one isotropic and one anisotropic fluid phase, the S-layer protein adsorbed preferentially to the isotropic phase. However, 2D crystallization could be nucleated in the boundaries between the two phases, but proceeded mainly underneath the anisotropic phase. The FTIR-measurements clearly indicate that the protein crystallization leads to an increased order of the lipid acyl chains.

5.5 Applications

Not only the composition of the interfacial layer, but also the mechanical properties, e.g., the dilational viscosity, of the layer is important for the stability of emulsions and foams (MacRitchie, 1990; Prins and Bergink-Martens, 1992; Dickinson, 1999; Bos and van Vliet, 2001b). In particular, both surface and bulk rheology as well as the disjoining pressure of the thin lamellae determine the stability of foams (Dickinson and Stainsby, 1982; Bos and van Vliet, 2001b). Hence, in technical applications thickeners are often added. The mechanical properties of interfacial films can to a large extent be controlled by the intermolecular interactions. Protein stabilization of a foam is mainly due to protein–protein interaction and the destabilization is thought of as a disruption of these interactions according to the Gibbs-Marangoni effect discussed above in the beginning of section 5.4.

5.5.1 Role of Protein-Emulsifier Interactions in Real Food (and Pharmaceutical) Systems

How the basic principles defined in section 5.4 come into play in some typical applications.

Sarker et al. (Sarker et al., 1995) discussed the effect of the surfactant properties on the stability of interfacial films in foams. The addition of small amount of lysophosphatidylcholine (LPC) was found to increase the foam stability of β-lactoglobulin foams (Sarker et al., 1995). A further increase of the surfactant concentration led to a decrease of the foam stability. The surface tension versus molar ratio of LPC and β-lactoglobulin show an inflection point close to unity molar ratio, corresponding to the binding of the surfactant to the protein. No increase of foam stability was, however, observed for mixtures of Tween 20 and β-lactoglobulin, instead the stability decreased with increasing surfactant concentration (Coke et al., 1990). The same observations was made for the stability of an oil-in-water emulsion, where it was found that small amount of Tween 20 increased the rate of shear induced coalescence of β-lactoglobulin stabilized emulsion droplets (Chen et al., 1993). The marked reduction in surface shear viscosity even at low surfactant to protein ratios confirmed that loosening of the protein layer occurred. The effects of LPC on interfacial rheology showed that at low surfactant to protein ratios, an enhancement in the surface elasticity was found (Gunning et al., 2004), which could explain the observed increase in foam stability. The protein–surfactant complex is thought of being less surface active and a further increase of the surfactant concentration will lead to replacement of protein and protein surfactant complexes with surfactant at the interface (Coke et al., 1990; Krägel et al., 1995). The mobility of the protein in a protein stabilized thin liquid film, as measured with the fluorescence recovery after photobleaching technique (FRAP), increases at lower surfactant to protein ratio for Tween 20 than for LPC (Fig. 5.19). This was attributed to the stronger binding of Tween 20, compared with LPC, to β-lactoglobulin (Sarker et al., 1995) and will also explain why the foam becomes unstable at lower surfactant concentration when Tween 20 is used. The foaming properties of puroindoline from wheat was also found to be improved by the addition of LPC (Wilde et al., 1993). Once the surfactant concentration becomes large enough, the protein–protein interactions within the surface film will be prevented, the mobility increased and thus the foam stability decreased according.

The lipid binding activity of puroindolines can be exploited to counteract the foam damaging effects of lipids. Lipid binding proteins can sequester lipids and prevent their adsorption and subsequent destabilizing of protein foams (Clark et al., 1994b). These proteins are common in cereals and may play a role in foam stability in baked products and beer.

Ionic surfactant can also induce flocculation of protein stabilized emulsions and this is depending on the nature of the protein–lipid interaction as discussed by Chen and Dickinson (Chen and Dickinson, 1995a; Chen and Dickinson, 1995b; Chen and Dickinson, 1995c). An anionic surfactant, sodium lauryl ether sulphate (SLES), at sufficient concentration has been found to flocculate gelatine stabilized oil-in-water

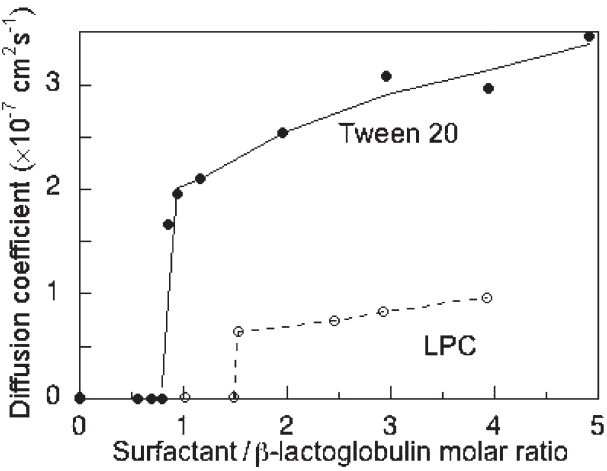

Fig. 5.19 The effect of surfactant addition on the lateral diffusion in the adsorbed mixed layer of surfactant and β-lactoglobulin, measured with the fluorescence recovery after photobleaching, FRAP, technique. The diffusion coefficients of the fluorescent probe 5-N-(octadecanoyl)aminofluo rescein and fluorescein isothiocyanate isomer 1 labelled β-lactoglobulin measured in the presence of L-α-lysophosphatidylcholine (○) and Tween 20 (●), respectively, are shown as a function of the molar ration between surfactant and β-lactoglobulin. The data are adapted from the work of Sarker et al. (1995) and Coke et al. (1990), respectively, in which the experimental details also are given

emulsion (Chen and Dickinson, 1995a). A further increase in surfactant concentration was found to lead to a restabilization of the flocculated emulsion. In bulk solution the anionic surfactant will, at high enough concentrations, cause precipitation of the positively charged gelatine. At a further increased surfactant concentration, the precipitate was redispersed. Gelatine was initially displaced by SLES from the interface (Chen and Dickinson, 1995c), but an increase of the surfactant concentration lead to an increase of gelatine concentration at the interface and the surface charge became partly neutralized (Chen and Dickinson, 1995b) causing flocculation. A further increase of the surfactant concentration lead to a decrease of the gelatine surface concentration (Chen and Dickinson, 1995c) and a restabilization of the emulsion (Chen and Dickinson, 1995a). It was also observed that the addition of SLES to a β-lactoglobulin stabilized emulsion not did cause any flocculation although some kind of complex was formed in bulk solution. It should be born in mind that β-lactoglobulin was negatively charged under the used experimental conditions. This confirms the electrostatic nature of the observed SLES induced flocculation of the emulsions stabilized by the positively charged gelatine. Flocculation of β-lactoglobulin stabilized emulsions was, however, observed in the presence of gelatine and SLES. Since it only occurred above the cmc of the surfactant it was suggested to depend on cross-linking of the emulsion droplets by surfactant micelles (Chen and Dickinson, 1995a).

Bylaite et al. found that emulsions with triglyceride oil generally proved to be more stable compared to those made with caraway essential oil as the dispersed phase (Bylaite

et al., 2001). However, the stability of the emulsions could be improved considerably by adding sb-PC. An increase in the protein concentration also promoted emulsion stability. Fang and Dalgeish arrived at a somewhat different conclusion for casein stabilized emulsions (Fang and Dalgleish, 1996). They found that the presence of DOPC destabilized casein stabilized emulsions of soybean oil in a 20-mM imidazole/HCl at pH 7.0. This seemed to be independent on whether DOPC was present during emulsification or if it was added to the emulsion as dispersed aggregates. At high concentration of casein, the emulsions were stable, and the decrease in surface load was a direct indication of the removal of casein from the interface by the presence of DOPC. The higher the DOPC concentration, the greater was the effect on emulsion stability and surface load. DPPC and egg PC either enhanced or did not affect the stability of the emulsion.

Waninge et al. (Waninge et al., 2005) studied the interaction between β-lactoglobulin and β-casein and milk membrane lipids at the oil-aqueous interface in emulsions. They found that the membrane lipid emulsified emulsions were dominated by the membrane lipids even after equilibrium with protein solutions. Protein displacement was not observed for β-lactoglobulin with time in contrast the displacement effects observed for the emulsions with β-casein, when both membrane lipids and β-casein were included during the emulsification. Based on results from three different types of emulsions, formed with different mixing order of the emulsifiers, they arrived on different alternative models that are described in Table 5.2. The eight different models can be divided into two main groups, where models I-III

Table 5.2 Models describing the oil–water surface with membrane lipids and β-casein/β-lactoglobulin. Adapted from Waninge et al. (2005)

Model *I*: A mixed monolayer including both protein and membrane lipids.	
Model *II*: A mixed monolayer with strong specific interactions between the protein and the lipid.	
Model *III*: A lateral separated monolayer.	
Model *IV*: A protein layer adsorbed on top of the lipid layer.	
Model *V*: A lipid monolayer formed at a protein layer.	
Model *VI*: A lipid layer adsorbed on top of a protein layer.	
Model *VII*: Vesicular aggregates attached at the interfacial protein layer.	
Model *VIII*: Vesicular aggregates immersed into the interfacial protein layer.	

are independent of mixing order describing an equilibrium structure and models IV are dependent on mixing order describing and therefore represent nonequilibrium structures. Based on the results obtained from the serum depletion method Waninge et al. could estimate the surface composition at the oil–water interface.

Model ***I-III*** assumes a mixed monolayer, which is expected to correspond to a coverage of 2–2.5 mg/m^2. Significantly higher total adsorbed amount was observed when the emulsion was prepared in presence of both protein and lipid. Furthermore strong effect of the mixing order was observed, which exclude models I-III. A structure corresponding to model ***IV*** is the only possible explanation for adsorption of protein to a membrane lipid emulsified emulsion but it may also occur when both components are emulsified together. However, the low protein adsorption (up to about 0.3 mg/m^2) observed when adding protein to the emulsion prepared in the presence of the lipid indicate a structure corresponding to model ***IV***.

For both the β-casein and β-lactoglobulin emulsified emulsions significant amount of membrane lipids were observed (around 1.4 mg/m^2) after adding the vesicles. Model ***V***, ***VI***, ***VII*** and ***VIII*** may describe the observed association between these emulsions and added membrane lipid vesicles. However, the pronounced hydrophilicity of the milk proteins makes a hydrophobic adhesion of a complete monolayer on top of the protein layer unlikely (Model ***V***). The fact that lipid adsorption is observed without a corresponding desorption of protein excludes model ***VIII***. Cryo-TEM images showed a few structures in agreement with model ***VII***, but the frequency was too low to fully explain the association observed. Model ***VI*** can be a result of a transition from model ***VII***, thus, a combination of model ***VI*** and ***VII*** seems to be the most likely structure in the system.

The observed gradual displacement of β-casein when emulsified together with the membrane lipids suggests the presence of the membrane lipid directly at the oil–water interface. Since the total adsorbed amount is well above monolayer coverage, model ***VIII*** appears more likely than model ***III***. However, it can be assumed that the system gradually transforms from a structure of type ***VIII*** over to the more simple structure of type ***III***.

The stable adsorbed layer when the emulsion is emulsified with both β-lactoglobulin and membrane lipids present suggests one of the structures ***VI***, ***VII*** or ***VIII***. However, model ***VI*** and ***VII*** seem more unlikely as the protein surface load is lower than the surface load of the pure protein emulsified emulsion. Notable is the absence of clear signs protein displacement. A possible explanation is that the protein layer is strongly crosslinked, as previously observed by (Chen and Dickinson, 1993; Chen et al., 1993; Mackie et al., 1999).

Several examples of how the properties of the oil phase composition can affect the structure of the adsorbed layer of protein on the emulsion droplet, and hence the stability of the emulsion, have been studied. For instance, the work of (Leaver and Dagleish, 1992) on the structure of adsorbed layers of β-casein on emulsion droplets, where it was found that the cleavage of the protein on the oil-droplet surface by trypsin gave different products depending on whether a triglyceride oil or tetradecane, was used. This demonstrates that the structure of the adsorbed layer depends on the composition of the oil.

5.5.2 Enzyme Activity and Protein/Emulsifier Application

What effect does the enzyme action have on the self-assembled emulsifier structure? How can lipase activity be affected by the presence of other proteins?

The action of lipolytic enzymes is of importance in a number of food applications or related areas, ranging from their use in detergents, as tools in modifying lipids to the breakdown of acylglycerides both as unwanted side effects and the naturally occurring process in the human intestine. It is well known that lipases work mainly at an interface and therefore one often speaks of "interfacial activation" in connection with lipase activity (Verger, 1997; Panaiotov and Verger, 2000). They are therefore an important example of lipid/protein interactions at interfaces. Lipases do play an important role in gastrointestinial tract for digestion of fat, (Patton and Carey, 1979), but they also have come to an increase use in industrial processes, including detergency and food processing (Svendsen, 2000).

Lipase act at such a low concentration that their presence as protein does not significantly affect the global lipid self-assembly structure. It is rather their catalytic activity that has an impact on the lipid self-assembly structure. It is also important to remember the action of lipases only decreases the time taken to reach the equilibrium and does not affect the equilibrium composition as such. Thus, the changes in structure in composition would have occurred even without the lipase if given enough time.

Here we will highlight some aspects in relation to the mechanisms of protein/lipid interactions. There are several types of lipases that act on phospholipids and triglycerides, but we will mainly discuss lipases catalyzing the hydrolysis of the ester bonds of triacylglycerols. The enzymatic activity is determined by the concentration of lipolytic enzymes associated with the lipid film and can be inhibited by various proteins (Gargouri et al. 1984a; Gargouri et al., 1984b). Experiments carried out with mixed protein/dicaprin films transferred over pure buffer yielded evidence that inhibition of hydrolysis was caused by proteins bound to the dicaprin film rather than by a direct interaction between protein and lipase in the bulk phase (Gargouri et al., 1985; Gargouri et al., 1986). Furthermore, since some lipases were inhibited by adsorption of proteins at the lipid layer, whereas other lipases were still able to hydrolyze a mixed protein/phospholipid layer, indicating that the inhibition of some lipases cannot be attributed merely to steric effects hindering accessibility to dicaprin molecules within the film. Surface concentration measurements of inhibitory proteins showed that only 5–9% of the area of a mixed lipid/protein film was covered by inhibitory proteins, implying that long-range electrostatic forces are likely to be involved in the inhibition as well as parameters such as surface viscosity and surface potential. However, similar inhibitory effects caused by melittin (pI > 10) and β-lactoglobulin A (pI = 5.2) at pH 8.0 strongly suggest that the nature of the inhibition is not an electrostatic phenomenon, but might be assigned to the effect on the properties of the hydrocarbon moiety of the lipid (Gargouri et al., 1987; Gargouri et al., 1989; Piéroni et al., 1990). The correlation between inhibition of lipase activity and the ability of the inhibitory protein to penetrate into the phospholipid monolayer support this suggestion.

In a simple experiment Wallin and Arnebrant demonstrated that a cubic phase was much faster decomposed by the action of lipase from *Thermomyces* (former *Humicola*)

lanuginosa than the reference sample consisting of triolein and aqueous phase (Wallin and Arnebrant, 1994). This was attributed to the much larger interfacial area in the cubic phase. In an in vitro study of lipolysis of triglycerides in a intestinal-like environment, Patton and Carey observed (Patton and Carey, 1979), apart from the initially occurring crystalline phase, a viscous isotropic phase composed of monoglycerides and fatty acids, which is identical to the one formed in monoglyceride systems. In excess of bile salts, the lipolysis products are rapidly solubilized in mixed micelles. However, the bile acid amounts in vivo are not sufficient to solubilize all lipids after a meal rich in fats, which implies that the liquid crystalline phases exist *in vivo* (Lindström et al., 1981). Lipase and water must be free to diffuse through the phases formed by the lipolysis products, surrounding the diminishing fat droplet. Thus, the bicontinuity as well as the incorporation properties of the cubic monoglyceride phases are thought to be important features for the lipolysis process (Patton et al., 1985). Borné et al. has in a series of studies investigate the affect of lipase action on liquid crystalline phase as well as other self-assemble structures such as vesicles and cubosomes (Borné et al., 2002a; Borné et al., 2002b; Caboi et al., 2002). Some of their findings are summarized in Fig. 5.20, which shows a schematic representation of the change in structure of the different liquid crystalline phases as a function of time after adding *Thermomyces lanuginosa* lipase. The observed changes in self-assembled structures could be predicted from either the monoolein –oleic acid-aqueous ternary phase diagram, where the lipolysis give rise to a transition of cubic → reversed hexagonal → micellar cubic → reversed micellar phase + dispersion or monoolein –sodium oleate-aqueous ternary phase diagram, where the corresponding sequence is lamellar → normal hexagonal. These difference in reaction sequences could be rationalized in terms of differences in degree of protonation of the fatty (Borné et al., 2002a). The initially lamellar phase had a high pH (about 10), that is a low degree of protonation and thus the degradation as expected follows monoolein–sodium oleate-aqueous ternary phase diagram. The initially cubic and hexagonal phase had low pH (4–7), that is a high degree of protonation and thus the degradation as expected follows the monoolein –oleic acid-aqueous ternary phase diagram. Adding *Thermomyces lanuginosa* lipase to aqueous dispersions of cubic phases (cubosomes) and lamellar dispersions (vesicles) at high water content and gave the corresponding morphological changes as for the liquid crystalline phases (Borné et al., 2002b). The phase diagrams of the relevant systems can thus be used as maps to navigate through the changes in the self-assembly structure of the substrate and the product. Borné et al. found similar specific activity of *Thermomyces lanuginosa* lipase on the cubic phase as on the reversed hexagonal monoolein based liquid crystalline phases, which was somewhat unexpected (Borné et al., 2002a).

5.5.3 New Products and Concepts of Using Protein/Emulsifier Interactions

Food nanotechnology and delivery of functionality.

The monoolein-aqueous system is thoroughly studied example of nanostructured system, where two types of cubic phases have been observed on the water-rich side

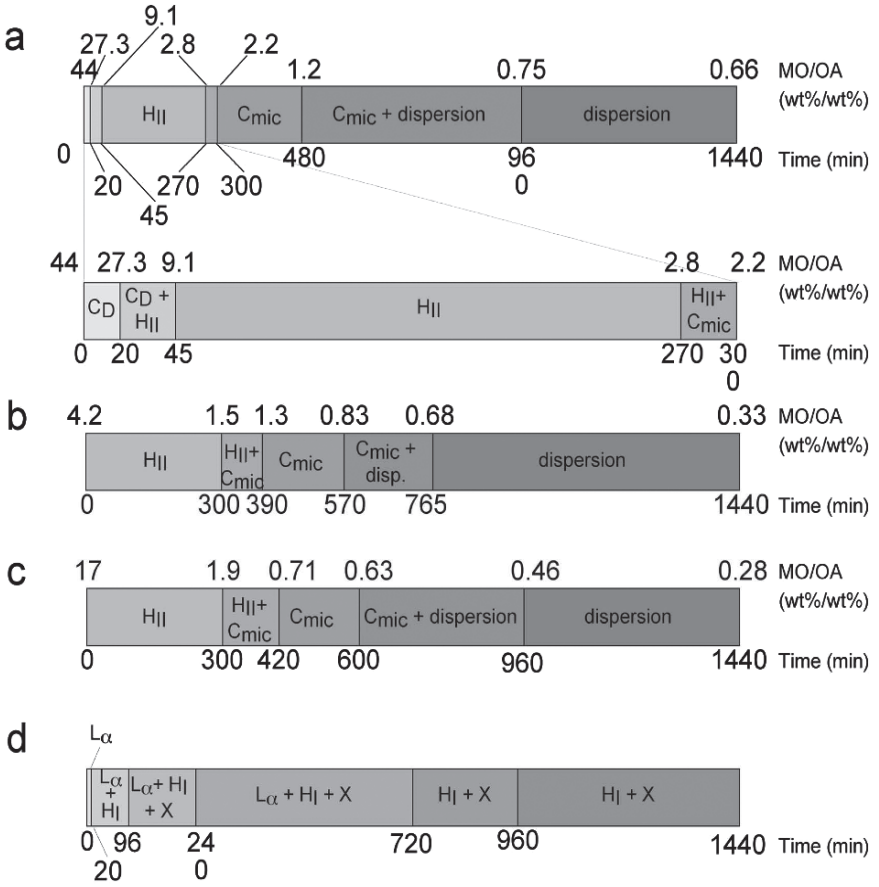

Fig. 5.20 Schematic representation of the change in structure during lipolysis of monoolein (MO) (or diolein DO) in different lc phases: (**a**) C_D phase (63 wt% MO, 37 wt% ^2H_2O), (**b**) Oleic acid (OA)-H_{II} phase (65.4 wt% MO, 15.6 wt% OA, 19 wt% ^2H_2O), (**c**) DO-H_{II} phase (68 wt% MO, 18 wt% DO, 14 wt% ^2H_2O) and (**d**) L_α-phase (10 wt% MO, 5 wt% Sodium oleate (NaO), 85 wt% ^2H_2O). The main liquid crystalline phases as determined by small angle X-ray diffraction (SAXD), are indicated in the figure as diamond type of bicontinuous cubic phase, space group Pn3m, (C_D), reversed hexagonal phase (H_{II}), normal hexagonal phase (H_I), lamellar phase (L_α) and micellar cubic phase, space group, Fd3m (C_{mic}). These may exist in excess of water or in the presence of minor amounts of other phases. Some of the observed reflections in the diffractograms, obtained by SAXD, could not be unambiguously assigned to a structure. This unidentified structure is denoted X. Figure adapted from Borné et al. (2002a), where details are given

of the lamellar phase (Larsson, 1983; Hyde et al., 1984; Landh, 1994; Briggs et al., 1996; Qui and Caffrey, 2000). Here we will highlight some of the main features that are of importance for the functionality and application of lipid-based liquid crystalline cubic phases. First it is the bicontinuity of the cubic phase. This is illustrated

Fig. 5.21a and b, where the mobility of glucose solubilized in the aqueous channels and vitamin K, solubilized in the lipid bilayer, respectively is illustrated. Figure 5.21a shows the concentration profiles of glucose in the cubic monoolein-aqueous phase equilibrated against water as determined by holographic laser interferometry (Mattisson et al., 1996). These profiles could be fitted to Ficks 2nd law, which gave a diffusion coefficient 4 times lower than the value in aqueous solution. The mobility of the molecules in the aqueous channels of the cubic phase is certain to be affected by the dimensions of the channels and the size of the solute. Thus, electrochemical studies of the transport of cytochrome c in the monoolein-aqueous cubic phase gave values of diffusion coefficients that were about 70 times lower than the bulk values (Razumas et al., 1996a). Figure 5.21b shows the mobility of monoolein and vitamin K_1, dispersed in the lipid bilayer as the NMR self-diffusion coefficients plotted versus lipid volume fraction in the cubic phases. It is noteworthy that the mobility of the introduced vitamin K_1 follows that of monoolein, indicating complete dispersion of vitamin K_1.

The dimensions of the water channels in the bicontinuous cubic phases, which depend on the degree of swelling and type of cubic phase are in the same range as the size of proteins (cf., Barauskas et al., 2000). Furthermore, as liquid crystalline phases they are quite flexible structures. These features have triggered a number of studies, which have shown that a large range of hydrophilic proteins with molecular weights up to 590 kD can be entrapped in the aqueous cavity of the monoolein-aqueous cubic phases (Razumas et al., 1994; Leslie et al., 1996; Nylander et al., 1996; Razumas et al., 1996a; Razumas et al., 1996b; Barauskas et al., 2000). The entrapped proteins have been found to be protected in the cubic phase, with retained native confirmation (Ericsson et al., 1983b; Portmann et al., 1991; Landau and Luisi, 1993; Leslie et al., 1996; Razumas et al., 1996b) and some enzymes can be kept for a very long time (months in some cases), with retained activity, which is not possible in aqueous solution (Razumas et al., 1994; Nylander et al., 1996).

Spectroscopic data have revealed changes in the molecular organization of the lipids evoked by the presence of the protein. FT-IR measurements on the monoolein-cytochrome c aqueous system showed that the presence of cytochrome c increased the conformational order of the monoolein acyl chain and caused structural rearrangements in the polar head group region (Razumas et al., 1996a). These observations are in agreement with the decrease of the monoolein packing parameter on upon incorporation of cytochrome c, which was deduced from increase in unit cell dimension of the cubic phase as determined by small angle X-ray diffraction.

The cubic monoglyceride phases have also the ability to solubilize lipophilic proteins like A-gliadin from wheat (Larsson and Lindblom, 1982) and bacteriorhodopsin (Landau and Rosenbusch, 1996) as well as relatively large amounts of membrane lipids (Gutman et al., 1984; Nylander et al., 1996; Razumas et al., 1996b; Baruskas et al., 1999; Engblom et al., 2000) and other hydrophobic compounds of biological relevance (Caboi et al., 1997; Baruskas et al., 1999; Caboi et al., 2001). These compounds are most probably dispersed in the lipid bilayer region of the cubic phase. The cubic phases can be used to achieve unique delivery functionalities in food systems, e.g., to solubilize functional ingredients and nutrients and to control

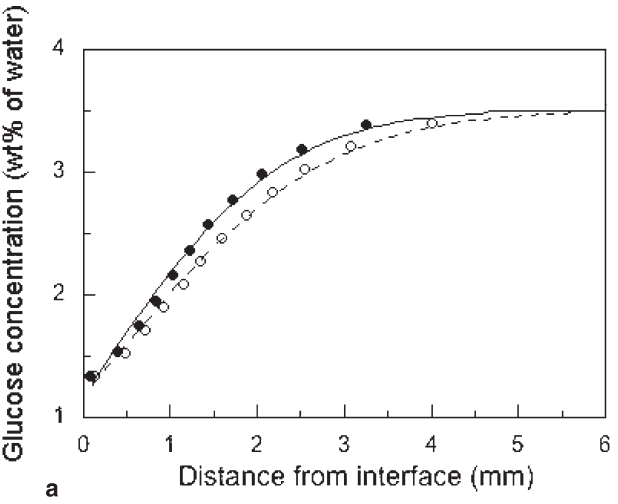

Fig. 5.21a Glucose concentration profiles in a monoolein- aqueous cubic phase (62:38 wt%), where the aqueous solution initially contained 3.5 wt% glucose, after 3 h (●) and 4 h (○) equilibration against pure water. The concentration is given as the wt% glucose in the aqueous solution of the cubic phase. The solid and broken lines are represent the best theoretical fit of Fick's law, giving diffusion coefficients of 1.39×10^{-10} m^2s^{-1} and 1.47×10^{-10} m^2s^{-1} after 3 and 4 h, respectively. The corresponding bulk value is 6.7×10^{-10} m^2s^{-1}. The data, obtained by holographic laser interferometry, are adapted from Mattisson et al. (Mattisson, Nylander et al., 1996; Nylander, Mattisson et al., 1996),where also the experimental details are given

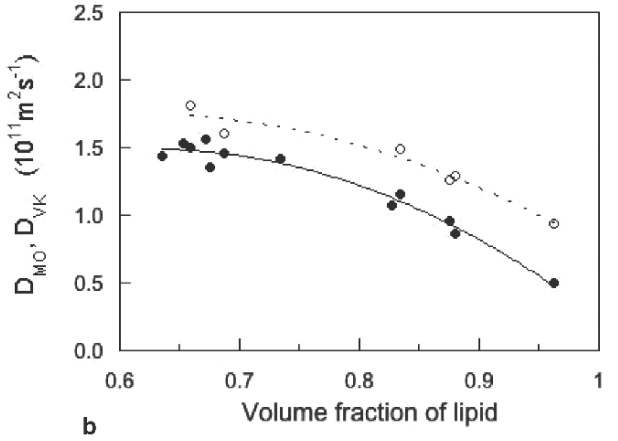

Fig. 5.21b NMR self-diffusion coefficients at 25 °C in monoolein-aqueous cubic phases containing 0–5 wt% vitamin K_1, are shown as a function of the lipid volume fraction (including vitamin K_1). The self-diffusion coefficients were measured in the cubic (both gyroid and diamond type) and in the reversed micelle, L_2, phases. Self-diffusion coefficients of monoolein (D_{MO}) (●) and vitamin K_1 (D_{VK}) (○) are shown. The lines are arbitrary fits to demonstrate the similar trends. The data are adapted from Caboi et al. (1997), where also the experimental details are given

release of flavors. Other applications in food systems can be to protect molecules from chemical degradation, or to increase the yield in Maillard reactions (Sagalowicz et al., 2006a).

Razumas et al. demonstrated that cubic monoolein-aqueous phases, containing enzymes, could be used as the biocatalytic layer in amperometric and potentiometric biosensors (Razumas et al., 1994). Their results for biosensors, based on a variety of enzymes, show that the long-term stability decreases in the order lactate oxidase > creatinine deiminase > glucose oxidase > urease, that is basically in the order of increasing molecular weight. Also the cubic phases of other amphiphiles like ethoxylated fatty alcohols can be used to entrap glucose oxidase, to construct a simple glucose monitor (Wallin et al., 1993). Landau and Rosenbusch demonstrated that the bicontinuous phases based on monoolein and monopalmitolein could provide matrices for the crystallization of membrane proteins like bacteriorhodopsin (Landau and Rosenbusch, 1996). They pointed out that the use of these types of cubic phase is advantageous as they provide nucleation sites, as the membrane proteins can be dissolved in the lipid bilayer. In addition they support growth by allowing lateral diffusion of the protein molecules in the membrane.

The bicontinuous cubic structures have by virtue of their well defined porosity also a large potential in drug delivery systems (Larsson, 1994).Stable particles of lipid-aqueous cubic phases, Cubosome® particles, can also be produced for this purpose (Larsson, 1989; Landh, 1994; Larsson, 1994; Gustafsson et al., 1996; Gustafsson et al., 1997; Larsson, 2000). The stability of Cubosome® particles, formed in monoolein-H_2O-based systems, and the corresponding dispersed H_{II} phase (Hexosome® particles) in the monoolein-triolein-H_2O system was found to increase in the presence of an amphiphilic block-copolymer (polyoxamer) (Landh, 1994; Gustafsson et al., 1996; Gustafsson et al., 1997). Barauskas et al. have devised a method to prepare very monodispersed Cubosome particles® and they found it was possible to further controlling dispersion particle size and nanostructure by varying the amphiphile concentration, the amount of charged species, and salt content (Barauskas et al., 2005a). In fact they showed that it is possible to prepare a range of different nanoparticle dispersions of self-assembled lipid mesophases with distinctive reversed cubic, hexagonal, and sponge phase structures by tuning the lipid composition and a simple, generally applicable and scalable method (Barauskas et al., 2005b). Some of these structures are shown in Fig. 5.22. A strong correlation between the mesophase internal structure and the shape of the nanoparticles was observed. For example, monocrystalline cubic-phase nanoparticles tend to maintain the shape of the cube, hexagonal phase give the shape of a hexanon, while the highly disordered "sponge" phase structures, favor the spherical shape. Guillot et al. (2006) identified possible internally self-assembled phases that occur in oil-loaded monoglyceride-based nanoparticles that are dispersed in water. The internal structure of these particles could be change by changing the temperature transformating from hexosomes to emulsified microemulsions through micellar cubosomes (emulsified reversed discontinuous micellar cubic phase) within a narrow range of an oil/monoglycerides ratio.

Several studies on different type of dispersed liquid-crystalline nanoparticles (LCNP) have pointed on the potential of using these systems for drug delivery as

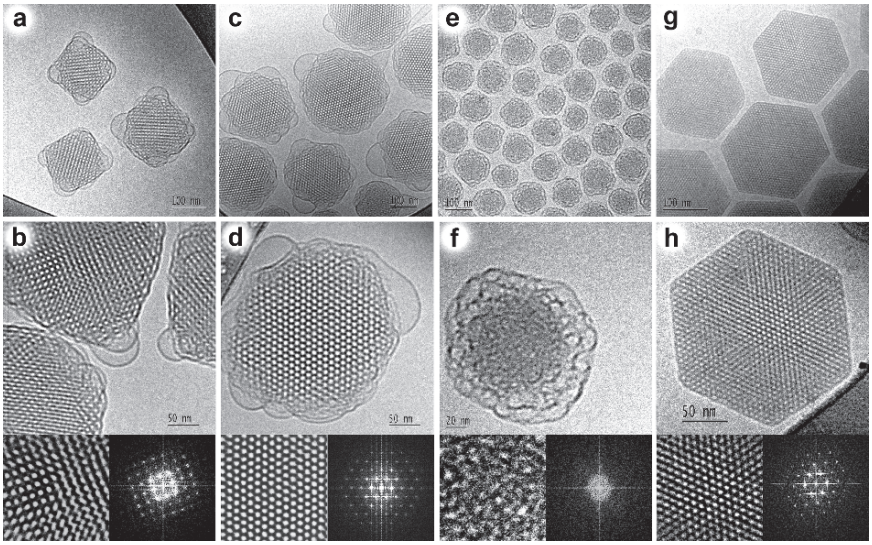

Fig. 5.22 Representative cryo-TEM micrographs of different nonlamellar lipid nanoparticles: Reversed bicontinuous cubic phase particles viewed along [001] (**a** and **b**) and [111] (**c** and **d**) directions. These dispersions were prepared at the weight ratio GMO/ F127/water) 1.88/0.12/98.0. Panels **e** and **f**: Monodisperse "sponge" phase nanoparticles prepared at the weight ratio DGMO/ GDO/P80/water) 2.13/2.13/0.74/95.0 (**e** and **f**). Reversed hexagonal monocrystalline particles made of lipids at the weight ratio DGMO/ GDO/F127/water) 2.25/2.25/0.5/95.0 (**g** and **h**). Fourier transforms of magnified areas in panels **b**, **d**, **f,** and **h** show the structural periodicity of the different nanoparticles consistent with the mesophase structures indicated above. The picture is kindly provided by Justas Barauskas and further details are in Barauskas et al. (2005b)

well as delivery of functionality to foods (Barauskas et al., 2005a; Barauskas et al., 2005b; Esposito et al., 2005; Spicer, 2005a; Spicer, 2005b; Almgren and Rangelov, 2006; Angelov et al., 2006; Barauskas et al., 2006a; Barauskas et al., 2006b; Boyd et al., 2006; Johnsson et al., 2006; Sagalowicz et al., 2006a; Sagalowicz et al., 2006b; Tamayo-Esquivel et al., 2006; Vandoolaeghe et al., 2006; Worle et al., 2006; Yaghmur et al., 2006). This have been shown, with both model and in vivo studies for the drug substance propofol; a well-known anesthetic agent currently used in clinical practice in the form of a stable emulsion (Johnsson et al., 2006). The propofol-LCNP formulation shows several useful features including: higher drug-loading capacity, lower fat-load, excellent stability, modified pharmacokinetics, and an indication of increased effect duration.

An interesting aspect of the interaction between liquid crystalline phases and proteins is the study of Angelova et al. (Angelova et al., 2005; Angelov et al., 2006). They showed that supramolecular three-dimensional self-assembly of nonlamellar lipids with fragments of the protein immunoglobulin gave bicontinuous cubic phase fragmented into nanoparticles with open water channels. These

so-called proteocubosomes are nanostructured open-nanochannel hierarchical fluid vehicles characterized by a cubic lattice periodicity of the lipid/protein supramolecular assembly (protein-loaded cubosomes).

5.6 Conclusion

The interaction between emulsifiers and proteins is to a large extent driven by electrostatic or hydrophobic interactions, or in many cases it is a combination of the two. Thus, it is commonly observed that ionic emulsifiers interact more strongly with proteins than nonionic ones. For emulsifiers with low water solubility, e.g., polar lipids, the interaction with proteins is largely dependent on the phase structure upon addition. The binding can, depending on the type of emulsifier, lead to stabilization of the protein structure at low-surfactant-to-protein ratios. However, an increase in surfactant concentration can induce unfolding of the protein and in some cases precipitation of the protein.

We have seen that the stability of emulsions and foams is determined by interfacial processes, which are affected by the properties of the interface as well as the interactions occurring in bulk solution. When no emulsifier/protein interactions are present, the composition of the interfacial film is determined by only the surface activity and concentration of the components. In the case of reversibility the most surface-active and/or abundant molecule dominates the interface and in the case of irreversibility the transport rate "the race for the interface" might also play a role. In this context it has to be born in mind that proteins can change their conformations (sometimes in a time-dependent way) at the interface. This may lead to a strong interaction between the protein and the surface, and multiple interactions between neighboring protein molecules. The latter has been found to hamper the displacement of a protein by more surface-active emulsifiers.

The presence of protein/emulsifier interactions can have pronounced impact on the interfacial behavior of the components. In cases where the emulsifier binding induces protein unfolding, exposure of hydrophobic domains of the protein, or precipitation at the interface due to charge neutralization, the surface activity of the complex is increased compared to the native protein. On the other hand, if the protein is more soluble or stabilized by the emulsifier interaction, the complex has a reduced tendency to adsorb at the interface. Precipitation of the complex in the bulk can cause loss of surface-active material and hence a decrease of the surface concentration. The emulsifier/protein interactions at interfaces can give more efficient packing and thus a higher total surface concentration. If protein/protein interactions take place at the interface, they may be disrupted by protein/emulsifier interactions.

Although emulsions and foams are stabilized by the same mechanisms, there are marked differences. First, there are profound differences between the two types of liquid interfaces: the liquid/air and the one between two condensed media. The oil/aqueous interface allows hydrophobic residues to become dissolved in and interact favorably with the oil phase, which is not possible at the air/water interface. It should

be noted that unfolding of the protein induced by action of emulsifiers or by the presence of an interface generally leads to exposure of hydrophobic residues; that is, the unfolded protein is more "oil soluble" than the native one. Second, in the stabilization of foams the viscoelastic properties of the surface film as well as the thin aqueous film have large effects. This means that protein/protein interactions in protein-stabilized foams are important, and the addition of surfactants can disrupt these interactions and lead to the collapse of the foam. On the other hand, low molecular weight emulsifiers can also stabilize the foam by means of Gibbs and Marangoni effects.

Steric and/or repulsive forces are important for stabilization of emulsions. Therefore, the mixed-protein/emulsifier layer should be optimized with respect to charge and/or by segments in the surface layer protruding into the aqueous environment to give a hairy structure that will sterically stabilize the emulsion.

This chapter has shown the enormous variety in emulsifier/protein interactions that can occur in food emulsions and foams. Each protein/emulsifier combination is unique and its behavior specific when applied in a particular foam or emulsion, where other ingredients are present. However, we have demonstrated that it is possible to establish certain principles for protein/emulsifier interactions. These principles based on mechanisms at the molecular level have also to be transferred to processes of manufacturing, storage, and distribution of food products based on emulsions and foams. Apart from the stability issues, other challenges are to increase the resistance of microbial growth without excessive use of antimicrobial substances, control digestion of the product, achieve controlled release of flavors as well as design new functional ingredients based on natural products.

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