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Competitive Adsorption between β-Casein or β-Lactoglobulin and Model Milk Membrane Lipids at Oil–Water Interfaces

RIANNE WANINGE,*,† PIETER WALSTRA,§ JAN BASTIAANS,# HANS NIEUWENHUIJSE,# TOMMY NYLANDER,⊥ MARIE PAULSSON,† AND BJÖRN BERGENSTÅHL†

Department of Food Technology, Engineering and Nutrition, Lund University, P.O. Box 124, S-22100 Lund, Sweden; Department of Agrotechnology and Food Sciences, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands; Department of Corporate Research, Friesland Coberco Dairy Foods, P.O. Box 87, NL-7400 AB Deventer, The Netherlands; and Department of Physical Chemistry 1, Lund University, P.O. Box 124, S-22100 Lund, Sweden

This study investigated the competitive adsorption between milk proteins and model milk membrane lipids at the oil−water interface and its dependence on the state of the lipid dispersion and the formation of emulsions. Both protein and membrane lipid surface load were determined using a serum depletion technique. The membrane lipid mixture used was a model milk membrane lipid system, containing dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, milk sphingomyelin, dioleoylphosphatidylserine, and soybean phosphatidylinositol. The model composition mimics the lipid composition of natural milk fat globule membranes. The interactions were studied for two proteins, β-lactoglobulin and β-casein. The mixing order was varied to allow for differentiation between equilibrium structures and nonequilibrium structures. The results showed more than monolayer adsorption for most combinations. Proteins dominated at the oil−water interface in the protein-emulsified emulsion even after 48 h of exposure to a vesicular dispersion of membrane lipids. The membrane lipids dominated the oil−water interface in the case of the membrane lipid emulsified emulsion even after equilibration with a protein solution. Protein displacement with time was observed only for emulsions in which both membrane lipids and β-casein were included during the emulsification. This study shows that kinetics controls the structures rather than the thermodynamic equilibrium, possibly resulting in structures more complex than an adsorbed monolayer. Thus, it can be expected that procedures such as the mixing order during emulsion preparation are of crucial importance to the emulsification performance.

KEYWORDS: Adsorption; β-casein; β-lactoglobulin; model milk membrane lipids; oil−water interface; lipid dispersion; emulsion

INTRODUCTION

Dairy products such as milk, cream, and evaporated milk are important examples of emulsions. The fat droplets in unhomogenized milk are covered with proteins and membrane lipids, a system known as the milk fat globule membrane (MFGM). The MFGM generally prevents aggregation and coalescence of the fat globules (*1*, *2*).

The native MFGM consists of a complex mixture of proteins and membrane lipids. The phospholipids in milk are present in the MFGM itself or in other membranous particles that most probably originate from the MFGM (*3*). The presence of vesicles as well as more complex morphologies has been shown in cream and buttermilk (*4*).

MFGM materials isolated from buttermilk have been used as emulsifiers (*5*, *6*). The emulsification properties have partly been attributed to the high protein content (*7*). Heat treatment of the cream and the industrial churning process appeared to affect the emulsifying behavior of MFGM proteins. MFGM isolate of this cream showed poor emulsification properties (*8*).

The formation of an adsorbed layer in the presence of both surface-active proteins and membrane lipids may result in competition between the two components. The lipid can produce a lower interfacial tension than the proteins, but its adsorption is hindered by its low molecular solubility. Proteins, on the other hand, are almost irreversibly adsorbed, partly due to the larger contact area per molecule and their ability to cross-link (*9*), and may be soluble in weak salt solutions.

* Corresponding author (e-mail Rianne.Waninge@livsteki.lth.se; fax +46 46 222 9517).
† Department of Food Technology, Engineering and Nutrition, Lund University.
§ Wageningen University.
# Friesland Coberco Dairy Foods.
⊥ Department of Physical Chemistry, Lund University.

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Malmsten (*10*) used ellipsometry to study protein adsorption onto phospholipid surfaces for human serum proteins interacting with various macroscopic phospholipid surfaces. He observed that most phospholipid and protein combinations gave very low lipid adsorption, but some, particularly anionic, phospholipids in combination with some proteins resulted in significant adsorption. This was also observed in emulsions analyzed with a serum depletion method (*11*). Competitive adsorption experiments using mixtures of β-casein or β-lactoglobulin with Tween 20 (polyoxyethylene sorbitan monolaurate) showed that Tween 20 completely (*12*−*15*) or partially (up to 60%) (*16*) displaced proteins from an oil−water interface. Anionic surfactants such as sodium dodecyl sulfate (SDS) also showed complete displacement (*17*). However, egg phoshatidylcholine (egg-PC) has been shown to partly decrease the amount of adsorbed β-casein at the surface when emulsified together (*18*). The casein adsorption at the surface did not change significantly for emulsions homogenized with whole casein in the presence or absence of phospholipids [dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), or egg-PC]. However, emulsions made with DOPC were less stable, and a lower proportion of casein adsorbed was obtained when the emulsion was stored for 48 h (*19*, *20*). In emulsions made with isolated MFGM and nonionic surfactants such as Tween 20, Tween 80, or Triton X-100 and β-casein or β-lactoglobulin, no displacement of the proteins was observed (*8*). However, these studies focused on the emulsification properties of the proteins of the MFGM, and no phospholipid surface loads were determined.

Our aim here is to describe the competitive adsorption between milk proteins and phospholipids at the oil−water interface and how the adsorption depends on the status of the lipid dispersion and the formation of emulsions. Therefore, we have determined both protein and membrane lipid surface load. The membrane lipids used were a model milk membrane lipid system, consisting of DOPC, dioleoylphosphatidylethanolamine (DOPE), milk sphingomyelin (SM), phosphatidylserine (PS), and soybean phosphatidylinositol (PI) (with respective weight ratios of 25.8:50:17.2:3.5:3.5), previously characterized (*21*). The model composition mimics the composition in natural MFGM materials (*3*, *22*).

MATERIALS AND METHODS

Materials. The simulated MFGM lipid mixtures were prepared by recombining a range of synthetic and natural lipids.

The phospholipids PI, dioleoylphosphatidylserine (DOPS), DOPE, DOPC, and SM were purchased from Avanti Polar Lipids (Avanti Polar Lipids, Inc., Birmingham, AL) and were used without further purification (99% purity).

β-Lactoglobulin and β-casein were supplied by INRA (Laboratoire de Recherché de Technologie Laitière, Rennes, France). The β-lactoglobulin was a mixture of the variants A and B with a purity of 99.4%. β-Casein had a purity of 90%. The freeze-dried proteins were dissolved in water or in a buffer, depending on the sample composition. The proteins were partly ^{14}C-labeled by methylation, as described elsewhere (*23*).

Miglyol 812 was obtained from CONDEA Chemie GmbH (Witten, Germany). Miglyol 812 is a medium-chain triglyceride oil (MCT oil) obtained by fractionation of coconut and palm kernel oil and contains mainly the fatty acids C8 (50−65%) and C10 (30−45%).

The water used was passed through a Milli-Q purification system (Millipore Corp., Bedford, MA). All other chemicals were of PA quality.

Sample Preparation: Vesicles. The polar lipid mixture of DOPC, DOPE, SM, PI, and DOPS (weight ratio 25.8:50:17.2:3.5:3.5) was selected to produce a composition resembling that of the natural MFGM. This mixture is referred to as "membrane lipids" throughout this paper.

Table 1. Composition of the Six Emulsions Prepared with 3 wt % MCT Oil

emulsion	protein (wt %) (β-casein or β-lactoglobulin)	membrane lipids (wt %)	added after emulsification
1	0.5		0.5 wt % membrane lipids
2		0.5	0.5 wt % β-casein or β-lactoglobolin
3	0.5	0.5	

The lipids were dissolved in chloroform in test tubes of soda glass. This procedure was used to ensure mixing on a molecular level. The chloroform was evaporated with a gentle flow of argon gas for 2 h. A 20 mM imidazole buffer with 50 mM NaCl was then added to the lipids. The final membrane lipid concentration in the emulsion was 0.5 wt %. Vesicles were prepared using a mini-extruder (Avanti Polar Lipids, Inc.). The lipid dispersion was pushed 19 times through two 200 nm polycarbonate filters.

Emulsion Preparation. Three types of emulsions were prepared with 3 wt % MCT-oil: (1) a protein (β-casein or β-lactoglobulin) emulsified MCT oil emulsion, to which membrane lipids were added as a vesicular dispersion; (2) a membrane lipid emulsified emulsion, where protein (β-casein or β-lactoglobulin) was added; and (3) a protein (β-casein or β-lactoglobulin) and membrane lipid emulsified emulsion with both emulsifiers present.

The compositions are shown in **Table 1**. Addition of 0.02 wt % sodium azide prevented microbiological growth during storage. The emulsification methods were similar for the three emulsions: 10 min of mixing using a high-shear mixer, Ystral X10/25 with a 10F tool (Ystral GmbH, Ballrechten-Dottingen, Germany) followed by 2 min of ultrasound treatment using an ultrasonic tip (Branson Sonifier B-12, Branson, Danbury, CT).

Experimental Procedure. The emulsions were prepared and divided into three samples. The first sample was analyzed immediately after preparation ($t = 0.1$ h). The other samples were transferred into sample tubes. The samples were slowly rotated during the 48 h of storage to prevent creaming. The sample tubes were completely filled to avoid exposure to an air−water interface during storage. Both of these precautions were necessary to prevent coalescence. The emulsions were analyzed at $t = 5$ h and at $t = 48$ h.

The amount adsorbed was determined by comparing the concentration of proteins and membrane lipids in the subnatant after centrifugation with the concentration in the complete emulsion. A mild centrifugation at 3000*g* for 30 min was used to separate the emulsions into a concentrated cream layer and a serum layer. The droplet-sized distribution of the emulsions was measured using a Coulter LS 130 without PIDS (Beckman Coulter, High Wycombe, U.K.). The emulsions were diluted with Milli-Q water in the apparatus to obtain a suitable absorbance. At least five runs per sample were performed. In addition, the emulsions were examined in an optical microscope.

The adsorbed amount of protein or membrane lipids was calculated by taking the difference in relative protein or membrane lipid reading in the whole emulsion and the subnatant and adding a correction term for the excluded volume (eq 1). The surface load was obtained by relating the adsorbed amount to the total surface in the emulsion (eq 2). The surface area of the emulsion droplets was derived from the area-weighted average droplet size (d_{32}) (eq 3).

$$c_{\text{adsorbed}} = c_{\text{emulsion}} - c_{\text{continuous phase}} \times (1 - \varphi) = c_{\text{emulsion}}\left[1 - \frac{i_{\text{subnatant}}}{i_{\text{emulsion}}} \times (1 - \varphi)\right] \quad (1)$$

In eq 1 c_{adsorbed} is the adsorbed amount expressed as a concentration, c_{emulsion} is the concentration added to the emulsion, $c_{\text{continuous phase}}$ is the concentration measured in the continuous phase (subnatant), φ is the volume fraction dispersed phase, $i_{\text{subnatant}}$ is the intensity [from the counter (protein) or from the phosphorus analyses (membrane lipids)]

Table 2. Errors in Measured and Calculated Data

parameter	symbol	δ[a]	base for the estimated error
intensity during the protein analyses.	$i_{protein}$	1.4%	obtained from replicates in actual measurements (df = 28)
amount of protein adsorbed	$c_{adsorbed}$	0.08 mg/m^2	obtained from eqs 5 and 1
surface area	A	1.5%	obtained from replicates
amount of protein adsorbed	Γ	0.08 mg/m^2 + 1%	obtained from eqs 1, 2, 5, and 7
concentration of phospholipids	c_{pl}	2.2%	obtained from replicates during the establishment of the method (df = 9)
extraction yield		2%	obtained from gravimetric analyses during the establishment of the methods (df = 6)
amount of phospholipids adsorbed	$c_{adsorbed}$	0.15 mg/m^2	obtained from eqs 5 and 1
amount of membrane lipids adsorbed	G	0.15 mg/m^2 + 1%	obtained from eqs 1, 2, 5, and 7

[a] The error is expressed as percent if it is proportional to the magnitude (measured units or products (eqs 6 and 7)) or in absolute units if independent of the magnitude (differences) (eqs 4 and 5).

of the subnatant, and $i_{emulsion}$ is the intensity measured from the emulsion before separation.

$$\Gamma = \frac{c_{adsorbed}}{A} \quad (2)$$

In eq 2 Γ is the amount adsorbed and A is the oil−water interfacial area of the emulsion.

$$A = \frac{\varphi \times 6}{1000 d_{3.2}} \quad (3)$$

In eq 3 d_{32} is the area-weighted average of the droplet diameter.

Fat Extraction and Phospholipid Analysis. The concentration of membrane lipids was analyzed after a fat extraction using the modified Röse-Gottlieb method described by Walstra and de Graaf (*24*), with the addition of 1.5 wt % NaCl. The extracted lipid fraction was ashed following the AOCS official method (Ca 12-55), in china crucibles using ZnO as a carrier. The phosphorus content was measured spectrophotometrically, as a molybdate−phosphorus complex. A mixture of 0.07 mL/mL sulfuric acid, 6.25 mg/mL ammonium molybdate, and 5 mg/mL ascorbic acid was added to the samples. This complex gives a blue color, and the sample absorbance was read at a wavelength of 820 nm (*25*).

Protein Analysis. The relative protein concentration was determined by counting the specific radiation activity of the proteins in a β-counter (LKB Wallac 1219 Rackbeta, Turku, Finland), by adding 500 μL of labeled protein solution into 10 mL of scintillation liquid (EcoscintA, National Diagnostics, Atlanta, GA) in polycarbonate vials.

Analyses of Errors. The errors in the analytical procedures and their consequences for the final results have been analyzed on the basis of replicates in the experimental series or on replicates during the establishment of the procedures. The errors in the calculated parameters were estimated using standard procedures for the propagation of errors, assuming no correlation between the deviations in A and B

$$\delta_{(A+B)} = \frac{\sqrt{A^2\delta_{(A)}{}^2 + B^2\delta_{(B)}{}^2}}{A + B} \quad (4)$$

$$\delta_{(A-B)} = \frac{\sqrt{A^2\delta_{(A)}{}^2 + B^2\delta_{(B)}{}^2}}{A - B} \quad (5)$$

$$\delta_{(AB)} = \sqrt{\delta_{(A)}{}^2 + \delta_{(B)}{}^2} \quad (6)$$

$$\delta_{(A/B)} = \sqrt{\delta_{(A)}{}^2 + \delta_{(B)}{}^2} \quad (7)$$

where δ refers to the respective relative standard deviation.

The standard deviations in measured and calculated numbers are shown in **Table 2**. On the basis of the result of the error analyses, the precision in the protein data ranged from 0.08 to ~0.11 mg/m^2 and that for membrane lipids between 0.15 and 0.20 mg/m^2. We have considered stochastic errors (outliers) between replicates in the calculations. Irregular errors and systematic errors are more difficult to account for. We have used standards and double experiments to validate the methods. The symmetric experimental plan was designed to reduce the possibility of irregularities and systematic errors.

Cryogenic Transmission Electron Microscopy (Cryo-TEM). The samples for Cryo-TEM observations were prepared in a controlled environment vitrification system (CEVS). The chamber temperature was 30 °C, and the humidity was close to saturation to prevent evaporation from the sample during preparation. A small amount of the sample (5 μL) was put on a lacey carbon film supported by a copper grid and gently blotted with filter paper to obtain a thin liquid film on the grid (20−200 nm). The grid was quenched in liquid ethane (at its freezing point) and transferred into liquid nitrogen. The technique is described in detail by Bellare et al. (*26*). The vitrified specimens were stored under liquid nitrogen and transferred to a transmission electron microscope (Philips CM120 BioTWIN Cryo) equipped with a post-column energy filter (Gatan GIF 100), using an Oxford CT3500 cryoholder and its workstation. The acceleration voltage was 120 kV, and the working temperature was −180 °C. The images were recorded with a CCD camera (Gatan 791) under low-dose conditions. The defocus was ~1 μm.

The emulsions for the Cryo-TEM experiments were prepared using a Microfluidizer (Microfluics Inc., Newton, MA), operated at 350 bar homogenization pressure, allowing the emulsion to pass through the interaction chamber five times. Microfluidization was used to obtain droplets small enough to be observable with Cryo-TEM.

RESULTS

***β*-Casein Emulsions.** The competitive adsorption between β-casein and membrane lipids at the oil−water interface was investigated using three emulsions with different mixing orders (**Table 1**).

The particle size, the adsorbed amount of membrane lipids, and the adsorbed amount of protein were monitored for 48 h. **Figure 1** shows the results for the three emulsions.

Figure 1A shows the results for the β-casein-emulsified emulsion. The droplet size was found to be 1.9 μm and had not changed significantly after 5 and 48 h. The initially adsorbed amount of protein was 2.6 mg/m^2, which might correspond to a monolayer of β-casein. This is in agreement with earlier studies on soybean oil emulsions, for which amounts of ~2 mg/m^2 were found (*27*), whereas Smulders (*28*) found an adsorbed amount of 3.8 mg/m^2 in a similar system.

A significant adsorption or deposition of membrane lipids of 0.3−1.4 mg/m^2 was observed 5 and 48 h, respectively, after membrane lipids in vesicular form had been added to the β-casein-emulsified emulsion; 1.4 mg/m^2 corresponds to 26% of the total membrane lipids present in the dispersion. It is interesting to note that the β-casein load remained constant. The membrane lipid load, 1.4 mg/m^2, can be compared with a typical

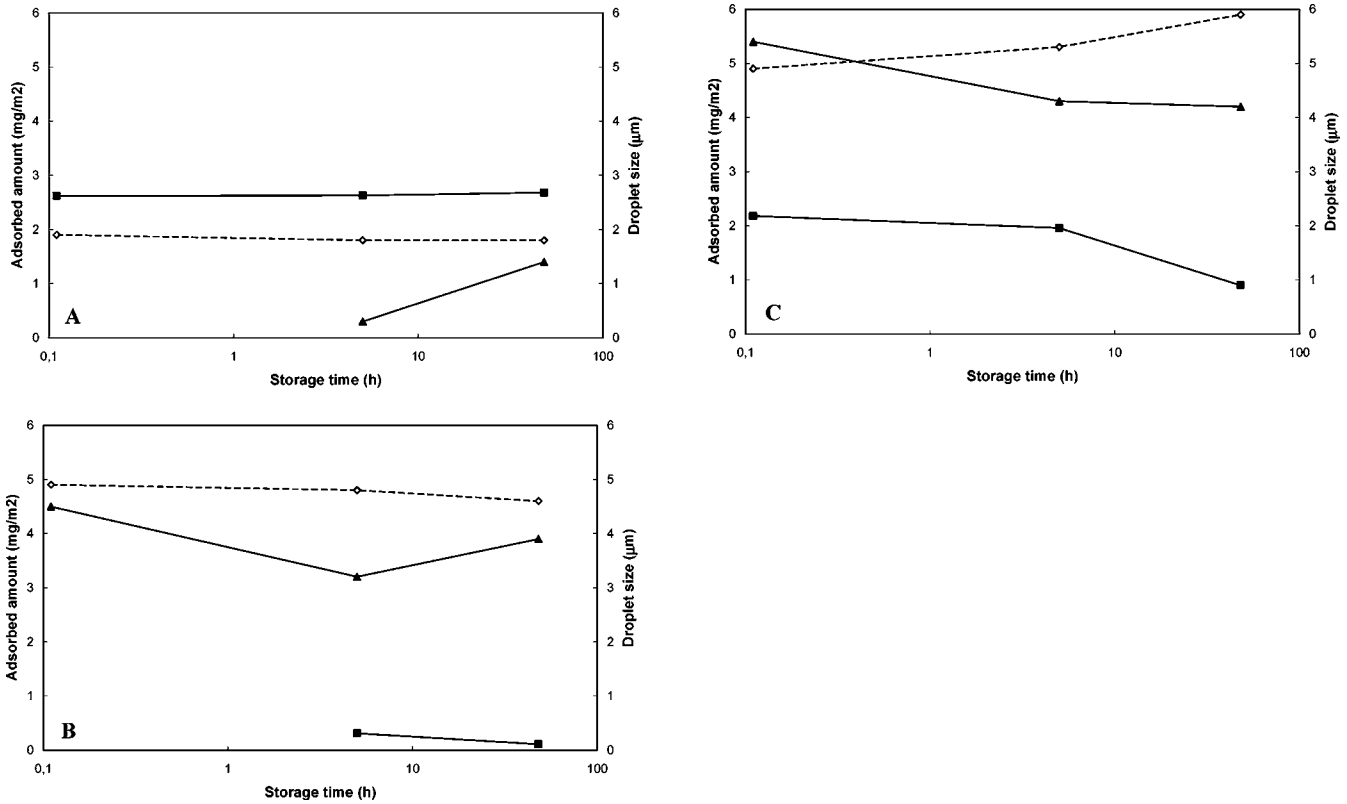

Figure 1. Droplet sizes (◇, μm), protein load (■, mg/m^2), and membrane lipid load (▲, mg/m^2) for (**A**) β-casein-emulsified emulsion with vesicular membrane lipids added, (**B**) membrane lipid emulsified emulsion with added β-casein, and (**C**) β-casein and membrane lipid emulsified emulsion.

monolayer of membrane lipids, which is between 2 and 2.5 mg/m^2 (based on X-ray data of bilayers of membrane lipids) (*29*).

Figure 1B shows the results from the membrane lipid emulsified emulsion. The particle size was larger than that of the casein-emulsified emulsion, ~4.6−4.9 μm. The emulsion remained stable during the experiment (although some scatter of particle size data was observed). The large size of the droplets was confirmed using light microscopy. The membrane lipid surface loads were rather high, ~4 mg/m^2, and equivalent to more than monolayer coverage. A figure exceeding monolayer coverage has also been observed previously. The formation of DOPC layers at an MCT oil−water interface, obtained by adding the phospholipids by dispersing them either in the oil or in the aqueous phase, was studied by ellipsometry (*30*). They found that adding the phospholipids to the oil phase formed multilayers, whereas adding through a vesicular dispersion in the aqueous phase resulted in a monolayer formation. Cruijssen (*31*) obtained a surface load of 2.4 mg/m^2 at the soybean oil−water interface with crude soybean lecithin, using the serum depletion technique. However, in this experiment all phospholipids present in the dispersion were associated at the surface of the emulsion droplets. In the present study the fraction of membrane lipids associated at the surface was between 50 and 39%, depending on the available interface. After 5 and 48 h, it was observed that the β-casein load remained very low, showing that the membrane lipid surface is a protein-rejecting surface.

The results from the β-casein and membrane lipid emulsified emulsion as a function of time are shown in **Figure 1C**. The droplet size immediately after formation was similar to the membrane lipid emulsified emulsion, but it increased slightly with time. The particle size data, confirmed by light microscopy, showed a bimodal distribution at $t = 0.1$ h and $t = 5$ h. After 48 h, it was observed that the small particles were absent. The membrane lipid surface load did not change greatly after 5 and 48 h and remained at a level corresponding to an average of about a bilayer (4−5 mg/m^2). However, the protein load decreased from 2 to ~1 mg/m^2.

It has previously been observed that zwitterionic phospholipids influence the emulsification performance when used as emulsifiers together with whole casein. The presence of DOPC destabilized emulsions with casein at pH 7.0. The destabilization occurred both when DOPC was present during the emulsification and when it was added to the emulsion as vesicles. The higher the DOPC concentration, the greater the effects on emulsion stability and surface load (*20*). Protein displacement was observed in a β-casein/soybean oil emulsion and in a β-casein/tetradecane emulsion when egg-PC was included before emulsification (*13*). The egg-PC surface load obtained was ~0.9 mg/m^2 with tetradecane and very low with soybean oil. The weak displacement observed with phospholipids is in contrast to the more pronounced displacement observed in several studies with water-soluble surfactants. Courthaudon et al. (*13*) observed total displacement of β-casein by etoxylated surfactants (Tween 20 and $C_{12}EO_8$) with *n*-tetradecane as the oil phase. However, when soybean oil was used, only half of the β-casein (from 2.3 to 1.2 mg/m^2) was displaced. It is interesting to note that when a 28-day-old emulsion was used, more protein remained at the surface (1.9 mg/m^2) (*16*).

***β*-Lactoglobulin Emulsions.** The experiments with β-lactoglobulin were performed as those with β-casein. The competitive adsorption between β-lactoglobulin and membrane lipids at the oil−water interface was investigated using three emulsions with different mixing orders (**Table 1**).

The particle size, the adsorbed amount of membrane lipids, and the adsorbed amount of protein were monitored for 48 h. **Figure 2** shows the results for the three emulsions.

Figure 2A shows the data for the β-lactoglobulin-emulsified emulsion. The initial droplet size was 1.3 μm, and it appeared

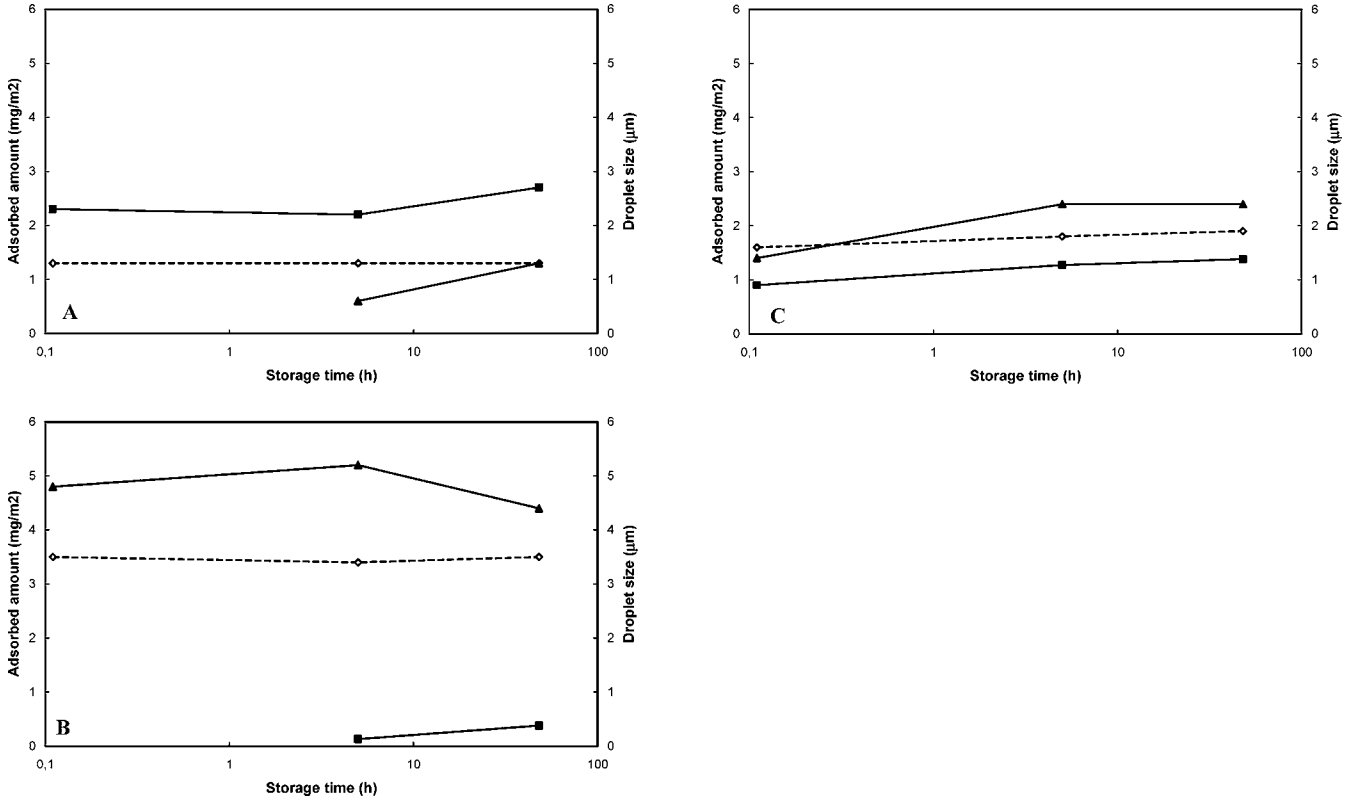

Figure 2. Droplet sizes (○, μm), protein load (■, mg/m^2), and membrane lipid load (▲, mg/m^2) for (**A**) β-lactoglobulin-emulsified emulsion with vesicular membrane lipids added, (**B**) membrane lipid emulsified emulsion with added β-lactoglobulin, and (**C**) β-lactoglobulin and membrane lipid emulsified emulsion.

to be constant with time. The adsorbed amount of β-lactoglobulin was 2.3 mg/m^2 and increased slightly after 48 h to ~2.7 mg/m^2. Previously reported data at oil−water interfaces show slightly lower results for the β-lactoglobulin load. Courthaudon reported 1.7 mg/m^2 from a tetradecane water emulsion (*15*). Smulders (*28*) found a similar β-lactoglobulin load (1.9 mg/m^2) onto soy oil droplets.

The membrane lipid load obtained was 0.6 mg/m^2 (t = 5 h) and 1.3 mg/m^2 (t = 48 h) after the membrane lipids were added in a vesicular form to the β-lactoglobulin emulsion. This result was comparable with that observed with the β-casein-emulsified emulsion.

In the membrane lipid emulsified emulsion, the droplet size was 3.5 μm and remained constant throughout the 48 h (**Figure 2B**). The adsorbed amount of phospholipids was 4.8 mg/m^2 and did not significantly change after the addition of β-lactoglobulin during the 48 h of storage. The low amount of β-lactoglobulin adsorbed, ~0.3 mg/m^2 after 5 and 48 h, is similar to the membrane lipid emulsified emulsion with added β-casein (emulsion 2). This result is in agreement with a previous study in which these proteins were added to vesicles with the same membrane lipid composition and in which a very low adsorbed amount of proteins (~0.1 mg/m^2) was observed (*23*).

In **Figure 2C**, the droplet size (d_{32}) of the β-lactoglobulin and membrane lipid emulsified emulsion 6 was 1.6 μm, increasing to 1.9 μm after 48 h. However, there were no signs of bimodality in the particle size distribution or of loss of a smaller particle fraction from the confirming light microscopy.

The amount of adsorbed phospholipids was 1.4 mg/m^2 at t = 0.1 h, increasing to 2.4 mg/m^2 after 48 h. The amount of adsorbed β-lactoglobulin increased slightly from 0.9 to 1.4 mg/m^2. β-Lactoglobulin is clearly less susceptible to displacement by the membrane lipids than β-casein in the mixed emulsion droplets. Displacement of β-lactoglobulin has previously been studied using micelle-forming surfactants. Partial displacement by Tween from the surface has been observed by de Feijter et al. (*17*), regardless of how the surfactant was added to the system.

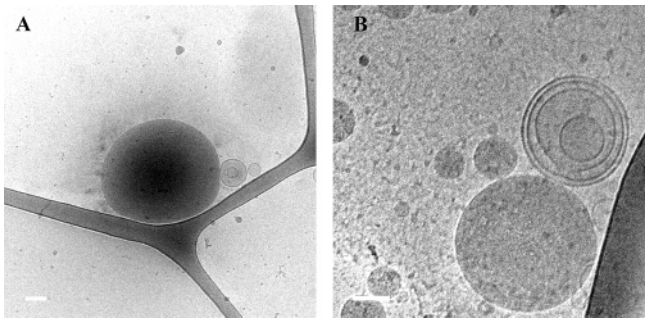

Figure 3. Cryo-TEM micrographs of (**A**) β-casein-emulsified emulsion with added membrane lipids as vesicles and (**B**) β-lactoglobulin-emulsified emulsion with added membrane lipids. Scale bar = 100 nm.

Cryo-TEM of β-Casein- and β-Lactoglobulin-Emulsified Emulsion with Added PL. Cryo-TEM images of the β-casein- and β-lactoglobulin-emulsified emulsion with added vesicles were taken (corresponding to emulsion 1 in **Table 1**). The emulsification was performed using a Microfluidizer to obtain emulsions with a sufficiently small particle size to allow the Cryo-TEM investigation. The mean particle size (d_{32}) for both β-casein- and β-lactoglobulin-emulsified emulsions was 0.4 μm. Membrane lipid vesicles were added to both emulsions and were examined after 16 h.

Examples of images are shown in **Figure 3**. The emulsion droplets appear as dark homogeneous particles, whereas the vesicles appear as lighter spheres with a clear dark wall. Mainly separate emulsion droplets and vesicles were observed in both

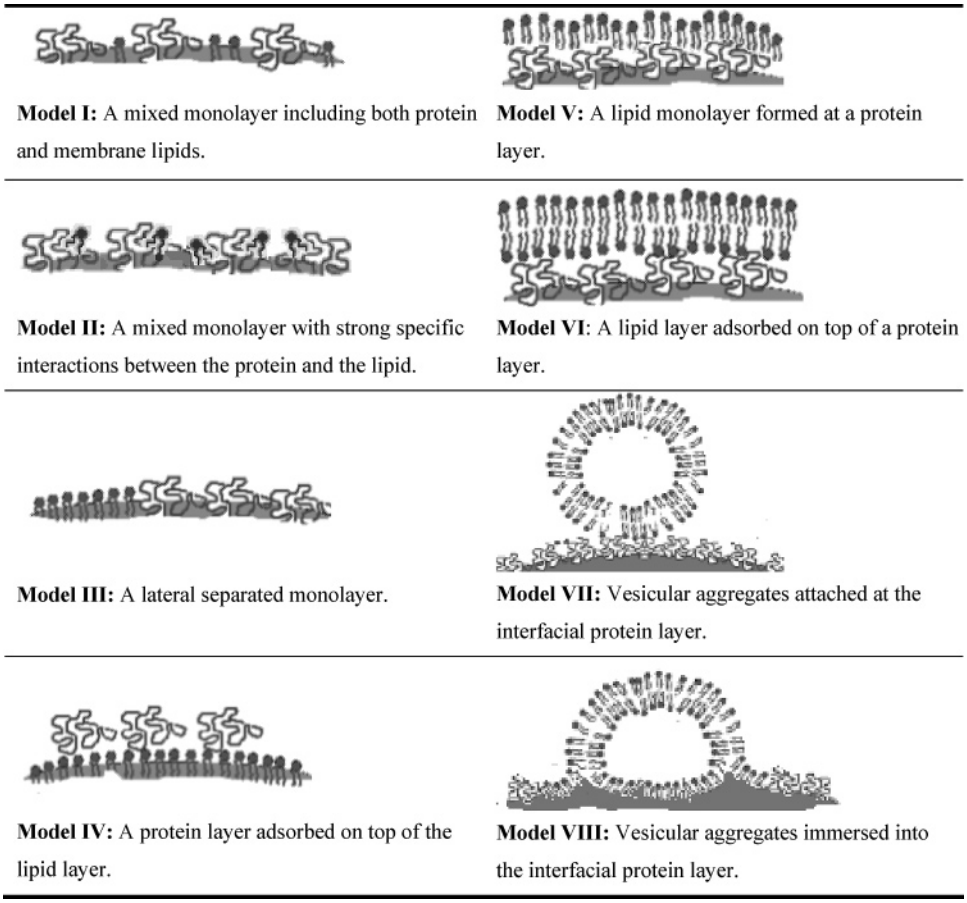

Figure 4. Models of different possible structures of adsorbed layers of proteins and membrane lipids at the oil–water interface.

emulsions, but in a few cases vesicles appeared to be associated to the emulsion droplets. No signs of adsorbed bilayers or multilayers of membrane lipids were observed.

DISCUSSION

Particle Sizes. Figures 1 and **2** show that the particle sizes (d_{32}) for the protein-emulsified emulsions are smaller than when protein and membrane lipids are emulsified, despite the greater ability of the membrane lipids to lower the interfacial tension. Agboola et al. (*32*) also found larger droplet sizes and bimodal distribution when soy lecithin was present in emulsion experiments using whey proteins as the prime emulsifier. These results differ from those obtained when water-soluble surfactants (micelle forming) were used together with β-casein. In this case smaller particle sizes were observed (*18*).

Micelle-forming emulsifiers have a high molecular solubility in water, whereas membrane lipids are dispersible in only vesicular form. It can be expected that the molecular solubility contributes, through dynamic exchange mechanisms, to the emulsification process (*33*, *34*). The membrane lipid mixture used forms uni-, bi-, and/or multilamellar vesicles (*35*). To obtain an adsorption layer, a vesicle should collide with an oil droplet in such a manner that phospholipids can spread over the oil−water interface. Thus, it is logical to assume that the membrane lipids have a much weaker ability to contribute to the emulsification than micelle-forming emulsions.

Surface Structures in Pure Membrane Lipid Emulsified Emulsions. The results show a surface load of ∼4−5 mg/m^2 (**Figures 1B** and **2B**), corresponding to twice monolayer coverage. Bilayer coverage seems very unlikely, as it would mean hydrophilic groups adhering to a hydrophobic surface. We may imagine that either an extra bilayer occurs in patches or that some emulsion droplets have a closed extra bilayer or even multilayer coverage. The latter would correspond with the coexistence of multilamellar and unilamellar vesicles that can be observed in membrane lipid dispersions (*35*). This observation would correspond with Friberg's idea of the presence of a third layer at the oil−water interface (*36*−*40*). Westesen et al. (*41*) have shown the existence of triple layers in lecithin-stabilized vegetable oil emulsions using synchrotron radiation. Incidentally, it was also shown that the occurrence of a multilayer was not necessary for the emulsion to be stable.

Possible Structures of the Adsorbed Layer Formed with both Proteins and Membrane Lipids. In the main part of the experimental work, both membrane lipids and proteins have been present simultaneously. A wide range of associative or competitive surface structures can be imagined, as both components are highly surface active.

In **Figure 4** eight different models are presented, illustrating various structures of adsorbed layers that could be formed in the presence of membrane lipid and protein at oil−water interfaces. The models can be divided into two main groups—models independent of mixing order describing an equilibrium structure and models dependent on mixing order describing nonequilibrium structures. The assumed interactions and the compositions of the interfacial layer are first briefly described, and then we try to compare the models with the experiments.

Models Independent of Mixing Order. Model I assumes a molecular mixture of both components at the interface without strong intercomponent interactions. The surface composition should be ratio dependent. The component giving the lowest interfacial tension at the prevailing bulk concentrations would

generally dominate at the interface, and the adsorption will have a competitive character.

Model II assumes strong interactions between the two components at the interface, so the adsorption in model II has a noncompetitive character. The interactions between the components lead to a maximum in the surface load when both components are present at the interface.

In model III, lipid−lipid and/or protein−protein interactions are stronger than the protein−lipid interaction. A two-dimensional phase separation occurs at the interface. This structure may also occur as an intermediate stage toward an equilibrium situation, for instance, that of model I. The surface load will be between the surface loads of the individual components.

Models Dependent on Mixing Order. Model IV assumes that proteins adsorb at a lipid monolayer. This can be the equilibrium situation of models when the component with the ability to form the lowest interfacial tension between oil and water forms the layer closest to the oil. The water-soluble component adsorbs on top of this primary layer, keeping its equilibrium with the dissolved state. It is a noncompetitive adsorption, and no displacement of the lipid layer is assumed. The total surface load will be higher than monolayer coverage.

Model V assumes protein at the interface with a monolayer of lipids on top. The proteins should be sufficiently hydrophobic to adhere to the oil−water interface and to allow at the same time for a hydrophobic adhesion of the lipid layer. The adsorbed amount should be more than a monolayer, and the adsorption of the lipid should be noncompetitive in character. The structure has to represent a nonequilibrium structure as the membrane lipids have a greater ability to lower the interfacial tension.

In model VI a lipid bilayer is assumed to adhere to the protein layer involving interactions from the polar headgroups, such as electrostatic interactions. The total adsorbed amount of lipid has to correspond to at least one intact bilayer (4.5 mg/m^2). The structure should represent a nonequilibrium condition, with the same arguments as above, although somewhat more stable due to the more closed and cohesive character of a lipid bilayer compared to an adhering monolayer.

In model VII complete vesicles are assumed to attach to an intact protein layer covering the oil−water interface. Here the same adhesive interactions are assumed as in model VI. The development of the structure assumes no displacement of protein and allows an incomplete coverage of the interface. This structure may transform into that of model VI.

Finally, in model VIII, the vesicles are able to wet the underlying oil phase and penetrate through the protein layer. This model gives partial displacement of protein but allows a total surface load above the level corresponding to monolayer coverage. Structure VIII may gradually transform into that of model III, and it would in a similar way depend on the strong interfacial tension-lowering ability of the membrane lipids.

Comparison with Experimental Observations. Hoping to find out what model can describe the interfacial structures formed when protein and lipids both are present, we applied the three principal variants of the mixing order (**Table 1**).

The importance of the mixing order in the experiments shows that the structures are not (fully) determined by the establishment of equilibrium, but rather by the history of formation of the emulsion. Hence, kinetics is as important as the thermodynamic equilibrium. The model describing the structures should accommodate five key observations from our study:

(i) In all emulsions studied, the total adsorbed amount after 48 h was greater than that corresponding to monolayer coverage.

(ii) Proteins in solution adsorbed only sparingly at an oil−water interface covered with adsorbed membrane lipid (membrane lipid emulsified emulsion).

(iii) There was a significant association of membrane lipid material dispersed in the aqueous phase with an oil−water interface covered with an adsorbed protein layer (protein-emulsified emulsions).

(iv) Protein may be displaced over time if protein and membrane lipids were present during the emulsification (β-casein in the protein and membrane lipid emulsified emulsion).

(v) A relatively stable protein and membrane lipid layer may be formed when both components were present during the emulsification (β-lactoglobulin in the protein and membrane lipid emulsified emulsion).

The total adsorbed amount shows that we have no obvious cases of adsorption structures corresponding to a monolayer (models I−III) in the presence of both proteins and membrane lipids. The strong effects of the mixing order support this observation. A structure corresponding to model IV is the only possible explanation for adsorption of protein to a membrane lipid emulsified emulsion as the membrane lipids gives an oil−water interfacial tension of ∼5 mN/m, which is significantly lower than the 10 mN/m obtained in the presence of proteins. Structure IV may also occur when both components are emulsified together. However, the low protein adsorption observed in the membrane lipid emulsified emulsion shows that a structure corresponding to model IV may explain adsorbed amounts of up to only ∼0.3 mg/m^2.

Models V−VIII may describe the observed association between the protein-emulsified emulsions and added membrane lipid vesicles. However, most hydrophobic side groups of the adsorbed milk proteins will be in the oil, which makes a hydrophobic adhesion of a complete monolayer on top of the protein layer unlikely (model V). The fact that no protein displacement is observed excludes model VIII. The Cryo-TEM images show a few structures in agreement with model VII, but the frequency is too low to fully explain the association observed. A structure as proposed in model VI may be difficult to prove using Cryo-TEM. Model VI can be a result of a transition from model VII. Hence, a combination of models VI and VII seems to be the most likely structure.

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. Because the total adsorbed amount is well above monolayer coverage, model VIII appears to be more likely than model III. However, it cannot be ruled out that the system gradually transforms from a structure of type VIII 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, models VI and VII seem to be more unlikely, as the protein surface load is substantially lower than the surface load of the pure protein-emulsified emulsion. Notable is the absence of clear signs of protein displacement. This may indicate that the protein layer is strongly cross-linked. Effects due to interfacial cross-linking with β-lactoglobulin have previously been discussed (*42*−*44*).

The kinetics of the adsorption of membrane lipids at the protein-emulsified emulsions (**Figure 1A** and **2A**) as well as the protein desorption from the protein and membrane lipid emulsified emulsion (β-casein, **Figure 1C**) is very slow. The time scale seems to be over days rather than hours. This is very slow if we compare with the time scale for protein adsorption,

which is less than an order of a millisecond (ms) (*34*). Also, adsorption of micellar surfactants is very fast (milliseconds), as is surfactant-induced desorption of adsorbed proteins. Typical time scales observed are within a few minutes (*45*). The time scale is influenced by inhomogeneities in the protein layer and displays a lateral inhomogeneity that can be viewed using AFM (*46*). The time scale for adhesion of liposomes to a bare hydrophobic macroscopic surface has been characterized (*47*) and found to be in the range of minutes, which is orders of magnitude slower than from a comparable system with micelle-forming emulsifiers. Adsorbed liposomes may transform into adsorbed intact monolayers within minutes for more highly unsaturated phospholipids and very slowly for more saturated systems. In the presence of an already adsorbed protein film, as in our experiment, this process has to be significantly slower and hours to days seem to be reasonable. The sensitivity of the protein film to penetration of emulsifiers should also be linked to the strength of the protein film, for example, the protein−protein interactions. Thus, the much higher stability observed for the highly cohesive β-lactoglobulin surface layer could well be a consequence of the influence on the time scale of the desorption process rather than an influence on the direction of the process. Charges at the surface may drastically delay the process, and, as a consequence the presence of salts, particularly Ca^{2+} may enhance the process.

This study shows clearly that the kinetics aspects can control the structures rather than the thermodynamic equilibrium and that this may result in structures far more complex than a simple adsorbed monolayer. It can therefore be expected that procedures during emulsion preparation, such as the mixing order, are of crucial importance to the emulsification performance.

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