Multilayer Formation on a Curved Drop Surface**

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The adsorption behavior of single components in emulsion systems such as proteins or phospholipids at air/water or liquid/liquid interfaces can provide information on the kinetics and interfacial activity.[1] The development of new techniques has made it possible to extend these studies to multicomponent systems at liquid/liquid interfaces.[2] This is particularly interesting for food emulsion systems where a colloidal droplet is often immersed into another liquid phase. The interfacial properties of milky emulsions are governed by, for example, β-lactoglobulin; these emulsions are often thermodynamically unstable.[3] Thus the addition of the low molecular weight surfactants such as phospholipids interact with the adsorbed protein layer at the interface and thus function as a stabilizer or an emulsifier.[1] Such a model system can be created by using the pendent drop technique, in which a solution of α-dipalmitoylphosphatidylcholine (DPPC) in chloroform was pumped through a tube to the tip of a Teflon capillary, which was immersed in an aqueous β-lactoglobulin solution. The behavior of two components from different sides of the liquid/liquid interface was used to compare the situation with that in an emulsion.

From kinetic measurements of the coadsorption we obtained the time-dependent dynamic interfacial tension γ of the mixed layer. The change in the shape and size of the pendent drop with the adsorption time has been observed both through the use of the pure β-lactoglobulin as well as the mixture with phospholipid. After a certain time the drop began to shrink, and a "skinlike" film was formed at the concave curved part of its surface (Figure 1). The detailed characteristics of drop size and the time to the onset of shrinking (Table 1) depend upon the bulk concentration. At a higher protein concentration, the initial drop size must be reduced to avoid its collapse due to the faster adsorption. The

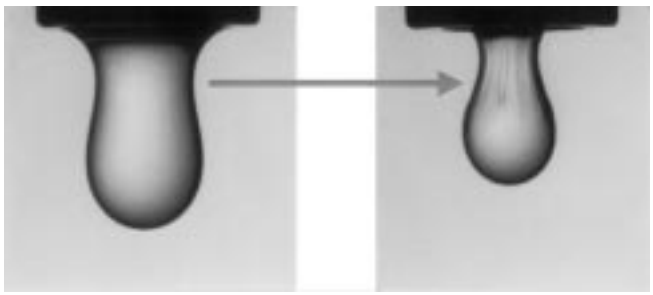

Figure 1. Critical change of the drop surface covered by a monomolecular adsorption layer or by a multilayer film.

Table 1. Drop size (V_i initial volume) and characteristic adsorption times of pure β-lactoglobulin at different concentrations. t_1: time to the onset of shrinking during adsorption; t_2: time required for the formation of the folded structure at the chloroform/water interface.

$c_{\beta\text{-lactoglobulin}}$ [mg L^{-1}]	V_i [μL]	t_1 [s]	t_2 [s]
0.6	15	5380	5670
1.2	15	3500	4300
1.8	13	2720	3150
2.4	12	2090	2600
3.0	12	1920	2430
3.6	12	1890	2270

shrinking began after 1890 s for the highest protein concetration of $c_{\beta\text{-lactoglobulin}} = 3.6$ mg L^{-1}, which is nearly three times faster than that for the lowest protein concentration. As a control experiment the system with a β-lactoglobulin concentration of 1.2 mg L^{-1} was selected for further studies of the coadsorption of the mixed phospholipid/protein system. The drop covered by pure protein started to shrink after 3500 s (Figure 2a and b), and had formed a skinlike surface after

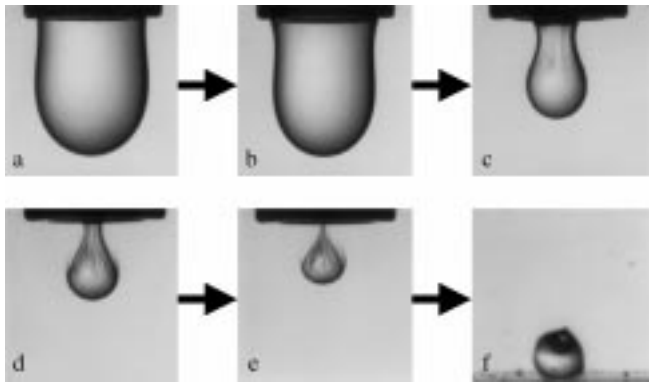

Figure 2. Images of drops covered by β-lactoglobulin or the mixed lipid/β-lactoglobulin at the chloroform/water interface after different adsorption times. a) A freshly produced β-lactoglobulin drop ($c_{\beta\text{-lactoglobulin}} = 1.2$ mg L^{-1}). b) After 3500 s the drop was covered by an adsorption layer of pure protein and started to shrink. c) A skinlike folded drop surface was formed after an adsorption time of 4300 s. d) On addition of DPPC ($c_{DPPC} = 1 \times 10^{-6}$ M), the folded drop surface formed already after 3000 s. e) After 4300 s the compressed drop collapsed at the edge close to the tip of the capillary. f) The dropped drop laid on the bottom of the cuvette like a ball.

4300 s (Figure 2c). This process was accelerated by the addition of DPPC to the chloroform solution ($c_{DPPC} = 1 \times 10^{-6}$ M) such that the folded surface had already formed after 3000 s. After 4300 s the compressed drop collapsed at the edge close to the tip of capillary (Figure 2d and 2e), and finally dropped to the bottom of the cuvette filled with protein buffer solution (Figure 2f).

On the basis of atomic force microscopy (AFM) measurements, we consider the formation of the skinlike folded surface (Figure 2c–e) as the formation of a multilayer along

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[**] This work was supported by the Max-Planck-Gesellschaft, the President fund of the Chinese Academy Science as well as the National Natural Science Foundation of China (NNSFC). We thank Marc Schneider for performing the atomic force mircoscopy (AFM) measurements and H. Möhwald (MPI, Golm) for critically reading the manuscript.

  1433-7851/01/4005-0891 $ 17.50+.50/0

the curved surface. For these measurements, we transferred the skinlike folded drop (Figure 2 f) to a substrate of mica at the bottom of the cuvette and removed the aqueous solvent by using a Hamilton pump and the organic solvent by evaporation. The remaining film was then examined by surface force microscopy on the top surface and the edge of the skinlike folded drop on mica (Figure 3 and 4).

Figure 3 shows the force micropscopy profile of a drop after drying, which was covered with a layer from adsorbed DPPC and β-lactoglobulin and had been transferred onto the mica before the folded surface formed (Figure 2 b). The surface morphology clearly reflects that a monolayer is formed. The maximum peak height is 1.7 nm and the diameter regions of aggregated molecules (bright areas) is 35 nm, suggesting that the dried protein was surrounded by phospholipids. The pure phospholipid monolayer has been previously determined to be 2 nm.[4] Therefore we estimate that the total thickness of the film is in the range of 4 – 5 nm.

Figure 4 C displays images of the scanned top surface of the dried drop (after formation of the fold) on mica. The maximum height of the peak is 1.8 nm and the width of the peak base is 35 nm, respectively. A comparison with Figure 3 A shows that here more protein is embedded in the phospholipid layers, indicating that a larger proportion of the protein is adsorbed at the interface and has mixed with the phospholipid. We believe that the two components interact with each other at the interface and stabilize the coadsorbed film. Otherwise gaps would be created by the aggregated protein units, which would have been observable in the three-dimensional (3D) image in Figure 4 C. Recent experimental work shows that the β-lactoglobulin is capable of penetrating phospholipid layers as a result of hydrophobic interactions.[5]

Furthermore, we have studied the fold structure at the edge to the mica substrate through stepwise measurements with the AFM tip. These revealed a thickness of 2.5 μm for the folded film, which indicates the presence of several superimosed layers (Figure 4 A); however, each layer is not identical. It is important to realize that one fold layer shown in the image does not represent a single monolayer since the transfer to the mica and the drying of drop could cause an inhomogeneous fold at the edge. Thus if we assume that one DPPC/β-lactoglobulin layer is 5 nm thick, the overall thickness of 2.5 μm represents a total of 500 layers. Therefore we deduce that the skinlike folding at the neck of the drop is the result of the formation of multilayers.

From previous experiments we have learned that the adsorption of pure DPPC at the same interface can lead to a change in the drop shape with an increasing bulk concentration.[6] However, a skinlike folded surface has never been observed even

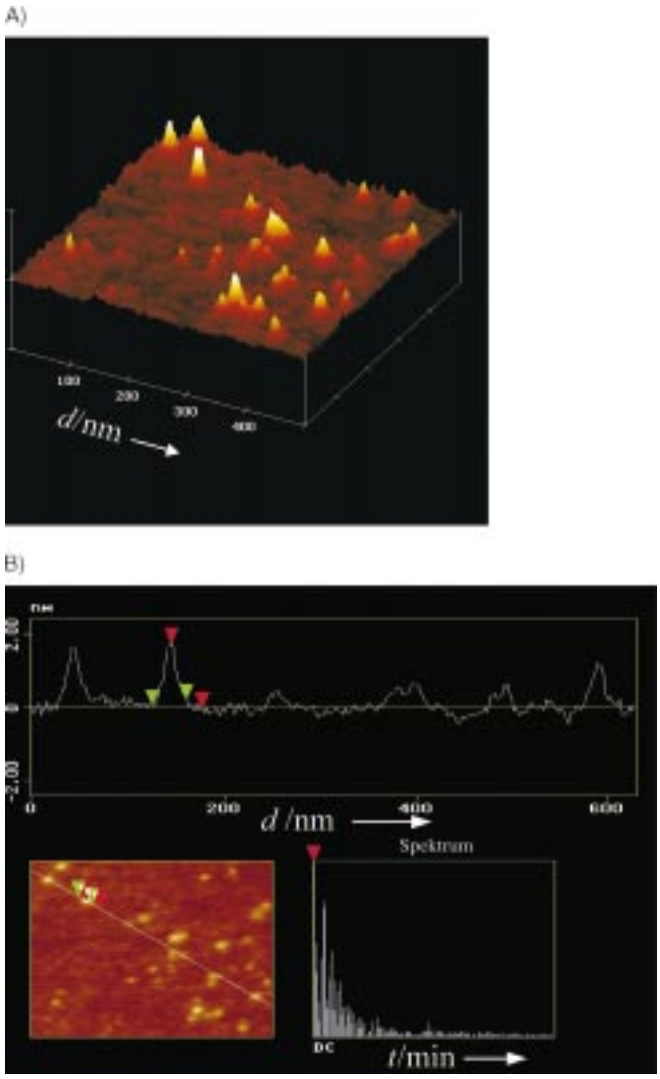

Figure 3. 3D AFM image (A) and height profile (B) of the complex DPPC/β-lactoglobulin film of an unfolded drop surface transferred onto mica. The transfer onto mica and the drying occurred after an adsorption time of around 3000 s.

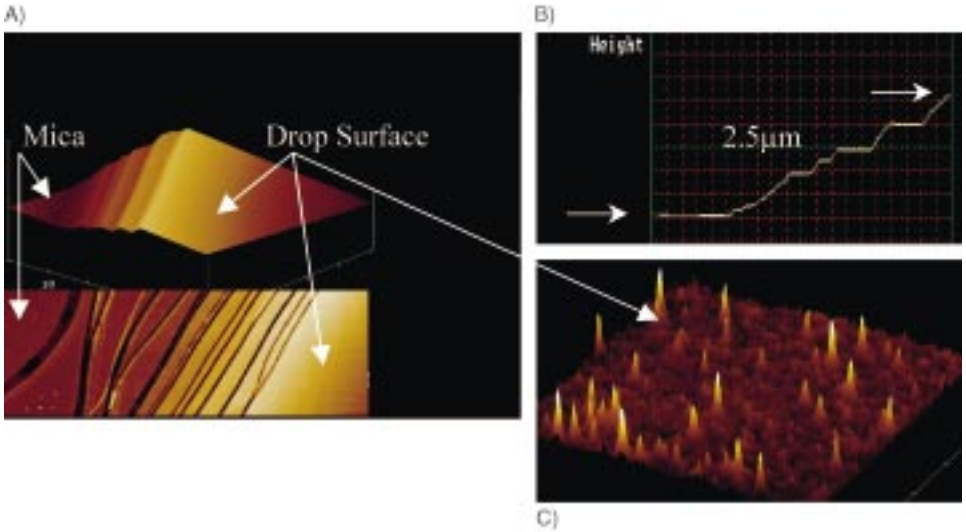

Figure 4. 3D AFM images (A, C) and height profile (B) of the complex DPPC/β-lactoglobulin film of a folded, skinlike drop surface transferred onto mica. The transfer onto mica and the drying occurred after the collapse of the drop.

  1433-7851/01/4005-0892 $ 17.50+.50/0 *Angew. Chem. Int. Ed.* **2001**, *40*, No. 5

for very high bulk concentration (above the critical micelle concentration (CMC)), at which a densely packed adsorption film covers the drop.

Detailed information on the properties of the films are essential for a better understanding of the results. Thus we performed experiments with different headgroups on the lipid and for different pH values of the aqueous phase. L-α-dipalmitoylphosphatidylethanolamine (DPPE) has the same chain length as DPPC, but a different headgroup (NH_3^+ instead of NMe_3^+). Under the same conditions, the adsorption of β-lactoglobulin in the presence of DPPE is faster than that in the presence of DPPC, as shown in the kinetics curves in Figure 5A. Accordingly, the time to the onset of the shrinking

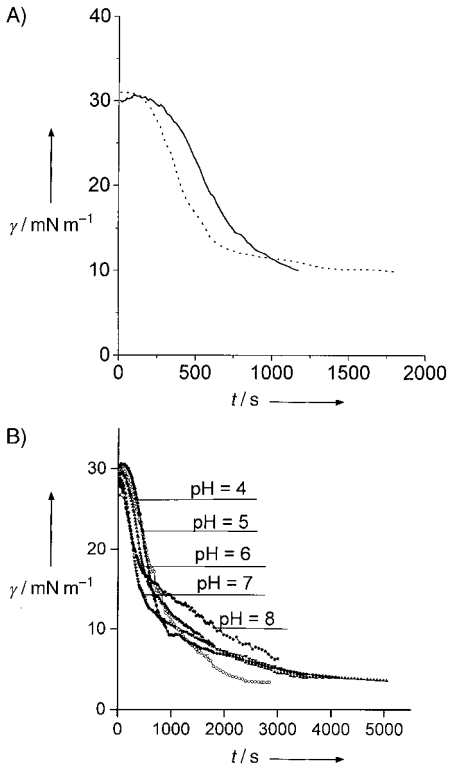

Figure 5. A) Kinetic curves (γ = interfacial tension) of the coadsorption of DPPC (solid line) and DPPE (dotted line) with β-lactoglobulin at the chloroform/water interface at room temperature ($c_{\beta\text{-lactoglobulin}} = 1.2\ mg\,L^{-1}$; $c_{lipid} = 1 \times 10^{-6}$ M); B) infleunce of the pH value on the kinetics of the coadsorption of the mixture of DPPC and β-lactoglobulin ($c_{\beta\text{-lactoglobulin}} = 1.2\ mg\,L^{-1}$, $c_{DPPC} = 1 \times 10^{-6}$ M) at room temperature.

of the drop is 200 s shorter with DPPE than with DPPC. The time for the formation of a skinlike folded surface is also shorter. As expected, the coadsorption kinetics of mixed lipid/protein influenced the rate of the formation of the fold on the surface of the drop and thus the formation of the multilayer.

DPPC is a zwitterionic lipid.[1, 7] Changes in the pH value of the aqueous phase can influence the charged state of β-lactoglobulin. The isoelectric point of β-lactoglobulin lies at pH 5.2.[1] Thus between pH 4 and 6 we expect no noticeable influence on the coadsorption behavior. In fact, the kinetic curves in Figure 5B show the predicted results. At pH values between 7 and 8 both the lipid and β-lactoglobulin carry a negative net charge, and the adsorption rates are similar. Figure 5B reveals that the adsorption at pH 7 or 8 is a little faster than at pH 4–6. This effect, however, is not particularly pronounced. This means that the coadsorption is predominantly controlled by the kinetics rather than by electrostatic interactions between the two compounds.

All the adsorption curves finally reach the same plateau, that is the adsorption acheives an equilibrium state. As expected, the pH value has only a very small influence on the rate of adsorption of the DPPC/β-lactoglobulin mixture, and also the shrinking time of the drop and the time for the formation of the skinlike folded drop surface structure are infleunced little by the pH value.

To summarize, a complex film can be constructed at a curved surface by the coadsorption of two components from different subphases. A pure β-lactoglobulin as well as the mixture of DPPC/β-lactoglobulin can form a skinlike folded film on a drop surface at the chloroform/water interface. The addition of DPPC accelerates the formation of the folded structure at the drop surface by coadsorption with β-lactoglobulin. The AFM analysis provided information on the morphology of the complex film and confirmed the formation of multilayer structures at the liquid/liquid interface.

The design of such complex films can be extended to the gas/water interface and to other two-component systems comprising a water-insoluble amphiphile and water-soluble surfactant. The ability of such systems to form multilayers facilitated the construction of a capsule with a polymer shell by the adsorption of the polyelectrolytes on colloidal templates,[8] which could be relevant for cosmetic and pharmaceutical applications.

In further work we will focus on the quantitative control of the composition and the structure of the mixed film as well as on the driving force which leads to formation of multilayers on a curved surface. With a smaller capillary with a diameter of about 1 μm it should be possible to form a bubble film.

Experimental Section

L-α-Dipalmitoylphosphatidylcholine (DPPC), L-α-dipalmitoylphosphatidylethanolamine (DPPE), and β-lactoglobulin (99% + purity) as well as chloroform (99% purity) were purchased from Sigma and used without further purification. The chloroform was saturated with Millipore water. The protein was dissolved in a 10 mM phosphate buffer solution. All experiments were performed at room temperature.

To measure the adsorption of the pure protein a chloroform drop of sufficient size was generated at the tip of a brass capillary, which was immersed in a solution of the protein in phosphate buffer.

For the determination of the coadsorption of lipid and protein, a few drops of a solution of the lipid in chloroform were initially allowed to drop to the bottom of a cuvette filled with aqueous protein solution, to form a saturated environment, and then a new drop was created. Lipid and protein molecules adsorb on the inside and outside of the chloroform/water interface of the pendent drop, respectively, and thus form a film. The development of the shape of the drop as a function of time was recorded with a CCD camera. Taking into consideration the adsorption time of the two components the interfacial tension γ was determined from the shape and size of the drop. The capture and analysis of the images of the pendent drop was carried out as described in reference [2]. The formation time and lifetime of the drop were recorded precisely by computer.

For the AFM measurements, a cleaned mica plate was carefully placed at the bottom of the cuvette. The cuvette was mechanically raised so that the

  1433-7851/01/4005-0893 $ 17.50+.50/0

mica plate touched the drop before the folding of the surface was established (Figure 2b). The aqueous solution in the cuvette was removed by using a Hamilton pump and the solvent inside was evaporated. The resulting film was studied by surface force microscopy. A similar process was used for the collapsed drop (Figure 2f).

Received: November 4, 1999
Revised: September 14, 2000 [Z14230]

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Chiral Molecular Recognition on Formation of a Metalloanthocyanin: A Supramolecular Metal Complex Pigment from Blue Flowers of *Salvia patens***

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Most blue flower color is the result of a metalloanthocyanin,[1, 2] a stoichiometric self-assembled supramolecular pigment consisting of six molecules of an anthocyanin, six molecules of a flavone, and two metal atoms.[3, 4] We have isolated various metalloanthocyanins from the petals of blue flowers and elucidated their structures. We have clarified the mechanisms of the development of blue color by chemical reconstruction of some supramolecules and structural analysis by NMR spectroscopy and X-ray crystallography.[1, 3] Very fine and strict molecular recognition,[5] including chiral molecular recognition, occurs during reconstruction. The aromatic chromophores in commelinin and protocyanin stack in a chiral manner, with anticlockwise self-association between two molecules of the anthocyanin or two molecules of the flavone, and clockwise stacking of anthocyanidin and flavone nuclei (co-pigmentation).[3] These phenomena must arise from the chirality of the sugars attached to the anthocyanidin and flavone units, although, no evidence has hitherto been provided in support of this conclusion.

Herein we describe the chiral molecular recognition on formation of the metalloanthocyanin protodelphin (**1**).[6] Elucidation of the gross structure of **1**, a genuine pigment from blue petals of *Salvia patens* (Figure 1), demonstrated

Figure 1. *Salvia patens.*

that it consists of the anthocyanin malonylawobanin (**2**),[7] the flavone apigenin 7,4′-di-*O*-*β*-D-glucoside (**3a**),[6, 8] and Mg^{2+} ions (Scheme 1). General and effective synthetic procedures for anthocyanins and/or flavones containing the optically antipodal glycosides are necessary for chiral recognition studies. We have developed a reliable glycosylation method for less-reactive phenols in flavonoids utilizing a new Lewis acid/base promoted glycosylation[9] with the peracetylglucosyl fluoride (**4**),[10] and succeeded in preparing natural apigenin 7,4′-di-*O*-*β*-glucoside **3a** and unnatural derivatives (partly) substituted with L-glucose (**3b**–**d**). Employment of these synthetic apigenin derivatives and natural **2** in the presence of Mg^{2+} ions allowed an examination of the chiral molecular recognition that occurs during formation of the blue complex pigment.

For the structural determination,[11] **1** was synthesized from the components according to our reconstruction method.[3] Compounds **2** and **3a** and Mg^{2+} ions were mixed in a weakly alkaline solution, then the mixture was purified by gel-permeation chromatography/liquid chromatography (GPC-LC)[12] to give pure protodelphin (**1**) in 61% yield. The UV/Vis spectra of the product were completely identical with those of the naturally occurring form[6] (Figure 2). Electrospray ionization mass spectrometry (ESI-MS)[13] of **1** gave a multiply charged molecular ion at m/z: 1751.8 ($[M-5H]^{5-}$) corresponding to the constitution $C_{396}H_{408}O_{222}Mg_2$ (average molecular weight: 8767.95, calcd 1751.37 $[M-5H]^{5-}$). The

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[**] We thank Mr. Y. Maeda (Chemical Instrument Center, Nagoya University) for help with molecular modeling, and the Ministry of Education, Science, Sports, and Culture of Japan (COE Research No. 07CE2004) for financial support.

  1433-7851/01/4005-0894 $ 17.50+.50/0