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Compression and shear surface rheology in spread layers of β-casein and β-lactoglobulin

Pietro Cicuta

Cavendish Laboratory and Nanoscience Center, J.J. Thomson Avenue, Cambridge CB3 0HE, UK

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Abstract

We investigate the surface viscoelasticity of β-lactoglobulin and β-casein spread surface monolayers using a recently discovered method. Step compressions are performed, and the surface pressure is measured as a function of time. This is a common experiment for surface monolayers. However in our experiments the pressure is recorded by two perpendicular sensors, parallel and perpendicular to the compression direction. This enables us to clearly measure the time relaxation of both the compression and shear moduli, at the same time, in a single experiment, and with a standard apparatus. β-Lactoglobulin and β-casein monolayers are interesting because of their importance in food science and because they exhibit universally slow dynamical behavior that is still not fully understood. Our results confirm that the compressional modulus dominates the total viscoelastic response in both proteins. Indeed for β-casein we confirm that the shear modulus is always negligible, i.e., the layer is in a fluid state. In β-lactoglobulin a finite shear modulus emerges above a critical concentration. We emphasize that in Langmuir trough dynamic experiments the surface pressure should be measured in both the compression and the perpendicular directions.

Keywords: Surface monolayer; Interfacial rheology; Stress relaxation; β-Casein; β-Lactoglobulin; Compression; Shear

1. Introduction

The stability and rheology of many complex fluids depends on the physical properties of interfacial films. Surfactant films act in two fundamentally different ways: they reduce the interfacial energy, and they confer viscoelastic properties to the interface [1,2]. Surface viscoelasticity is known to play a key role in slowing down or preventing droplet coalescence. Coalescence is a process that reduces the interfacial area, thus, if a film is present, it will either be compressed or the molecules will be forced out of the interface [1]. Both these effects require energy, and protein films provide a very efficient, cheap and bio-compatible stabilizing mechanism. Surface viscoelasticity is also one of the main factors controlling the mechanical behavior of the bulk system as a whole under shear. Although there has been ongoing research on the relation between surface rheology and bulk rheology [3], this is still an area that is not fully understood from either the experimental or modeling points of view.

Dairy products are an important area of food science, and in these products the milk proteins are the main surface active agents. Besides controlling coalescence and affecting the mechanical properties of the system, proteins also enable other physical processes like foaming, thickening and gelling. Two proteins are studied in this paper, chosen for their relevance in nutrition. They are each the most common representative of the two classes of milk proteins: caseins and whey proteins. The first protein, β-casein, makes up 25% by weight of the casein proteins, a class of proteins accounting for 85% of proteins in bovine milk. During cheesemaking, caseins are used to form the curd while the remaining proteins, that are soluble in the aqueous serum, are called the whey proteins. The second protein studied here is β-lactoglobulin and it accounts for 58% of whey proteins. Both β-casein and β-lactoglobulin are amphiphilic, and thus their presence plays an important part in determining the interfacial properties in milk-based systems [4]. Protein monolayers have been amongst the first films to be studied [5]. There exist extensive reviews on proteins at interfaces [4,6,7] and specifically on spread protein films [8]. These reviews focus mainly on adsorption isotherms and understanding protein

E-mail address: pc245@cam.ac.uk.

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conformation on the interface. The rheological behavior of protein films is still an open area of research.

For small deformations the mechanical response of the surface layer will be linear with the applied strain. The experiments presented here are in this linear response regime. There are two fundamental modes of mechanical deformation for a surface: shear (at constant area), and compression (at constant shape). The complete surface viscoelastic characterization involves determining both of these moduli. Each of these response moduli can have elastic and viscous character, so four parameters have to be measured. The focus in the present paper is on a new method for measuring the compressional and shear viscoelasticity of protein films.

2. Surface rheology on protein films

2.1. β-Casein and β-lactoglobulin

β-Casein exists in solution as an equilibrium between single chains and proteins aggregated to form micelles. It is believed to resemble a random coil chain (no secondary structure) of 209 residues and has a molecular weight of about 24 kDa. Its particularly remarkable emulsifying properties arise from the blocky distribution of hydrophilic and hydrophobic residues along its length. All of the phosphate groups are in the first 42 positions, making this end of the polymer a very hydrophilic tail.

β-Lactoglobulin contains 162 amino acids and has a molecular weight of about 18 kDa. The molecule contains two disulfide and 1 free sulfhydryl groups and no phosphorus, and compared to β-casein it is more globular and has a well-defined secondary structure. In solution in the bulk at room temperature β-lactoglobulin can be found in diversely aggregated forms, depending on the pH.

The charge distribution on the proteins can be estimated from the published primary sequence and the known dissociation constants for amino acids. For both β-casein and β-lactoglobulin this calculation shows overall neutrality at around pH 5, in agreement with published data. An overall positive net charge is found below this pH, and an overall negative charge above it. The net charge is estimated to be around −4 e at pH 8 and −9 e at pH 10 (e is the electron's fundamental charge unit).

Both these proteins unfold to a considerable degree when they adsorb at the air/water or oil/water interfaces [4]. In monolayers of these proteins, at low concentration, we found in previous work a polymer-like 2d semidilute regime, and we verifed that the mechanical properties of these semidilute systems are in many ways analogous to the behavior of monolayers made of neutral synthetic homopolymers [9,10]. β-Lactoglobulin was also previously studied at high concentrations, near close packing, where the layer is not fluid any more and exhibits a finite shear modulus [11,12].

This work focuses on the phenomenon of very slow (∼hours) stress relaxation. Similar very slow stress relaxation is seen in many polymer monolayers, and the reasons for it are not fully understood. This has been motivating a lot of recent work addressing polymer dynamics on slow timescales (frequencies around 1 Hz) [13,14].

2.2. Review of experimental work

A comprehensive overview of the vast experimental literature on protein films is given in the monograph by Mobius and Miller [4]. Regarding the proteins used in this work, initial work was done by Graham and Phillips [15,16]. They studied the adsorption behaviour of β-casein, and were able to show the relation between the amount of protein in the subphase, on the surface and the resulting osmotic pressure by combining radiotracers and ellipsometry. They also performed a subphase exchange experiment which demonstrated that an amount of β-casein equal to 2.6 ± 0.1 mg/m^2 is irreversibly adsorbed. This is a very good indicator of the amount of protein needed for complete coverage of the surface. Other key results have been obtained by neutron reflectometry [17] and ellipsometry [18] on β-casein at high surface coverages, showing that the protein adsorbs to the interface with a thin dense layer right at the surface and a thicker, less dense layer beneath it. It has been proposed that this sublayer is composed of the hydrophilic N-terminal end of the molecule. This structure makes β-casein layers very interesting, and leads to very special surface rheology. The protrusion onto the subphase of the N-terminal end was studied in recent work by our group [9], and is also supported by a number of experiments: X-ray and neutron reflectivity [19], proteolytic cleavage [20], Monte Carlo simulations [21] and self-consistent field simulations [22].

β-Lactoglobulin films have also been extensively studied, especially as adsorbed layers. Particularly relevant for comparison with the spread films investigated here are two experiments on the shear rheology of adsorbed layers [23,24].

2.3. Materials used in this work

β-Casein (Sigma, C-6905, bovine milk, min. 90% pure, lot. 25H9550) and β-lactoglobulin (Sigma, L-0130, bovine milk, mixture A and B types, min. 90% pure, lot. 91H7005) were used as supplied. 1 mg/ml solutions in deionised water were prepared from the dried, powdered proteins, stored in a refrigerator and used within 5 days.

These concentrated solutions were used to spread the proteins on the surface of aqueous solutions in a Langmuir trough (see Fig. 1). A micropipette was used to create droplets of volume around 0.5 μl, and these were deposited on the surface. This is one of the standard methods for spreading proteins. The other common method is to run a drop of the spreading solution down a rod that touches the surface. It is difficult to quantify exactly what fraction may dissolve into the subphase, but it is probably not more than 10%, since the area per protein at overlap agrees well with the expected gyration radius [9]. In the experiments presented here, 25 μl of the concentrated spreading solution were deposited on a 500 cm^2 trough.

To control the pH, phosphate buffer solutions were prepared with 0.01 M ionic strength using deionised water (Elgastat UHQ, Elga, UK) and Sigma-Aldrich Analytical Grade reagents. To control ionic strength, further 0.01 M NaCl was added. The final buffer pH were measured using an electronic meter (ATI Orion, USA) before use.

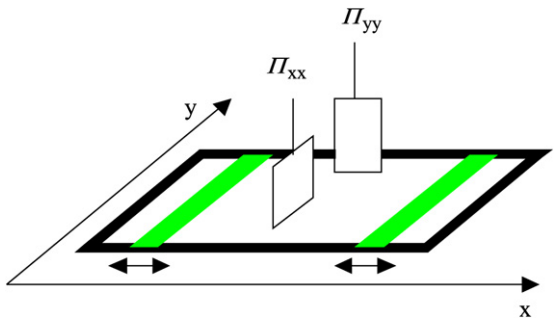

Fig. 1. Diagram of the Langmuir trough setup with symmetric compression barriers confining the monolayer film to the central area of the trough. Two pressure sensors record the surface pressure at the same time. The two sensors are maintained at 90°, and record pressures Π_{xx} and Π_{yy}, as indicated.

2.4. General remarks on surface moduli

The mechanical response to compressions is proportional to the compression elastic modulus ε. If the compression is very slow (quasi-static) then an equilibrium modulus is probed, which can be measured from the slope of a pressure–area isotherm:

$$\varepsilon_{\text{eq}} = -A\frac{\partial \Pi}{\partial A}. \tag{1}$$

If the compression speed is finite, then there might be friction resisting the compression flow, and the resistance is characterized by the compression (dilatational) viscosity η_{d}, defined as:

$$\eta_{\text{d}} = A\frac{\Pi - \Pi_{\text{eq}}}{\partial A/\partial t}. \tag{2}$$

For a complex material these parameters ε and η can be frequency dependent (i.e., they also depend on the history of deformation), and there are various ways in which to determine the frequency dependence. The most common experiments are to apply a sinusoidal deformation, or to apply a sudden "step" strain. In the limit of linear elastic response (valid for small enough deformations) these methods are equivalent. In recent work we studied β-lactoglobulin monolayers by oscillatory strain [11]. In this work we extend the systems investigated, and prove our surface rheology method by studying the response after a fast deformation.

If the sample is quickly strained compressively then, if it is viscoelastic, there will be an overshoot compared to the new equilibrium pressure Π_0, and then the pressure will relax to the equilibrium value. We can write this relaxation as:

$$\Pi(t) = \Pi_0 + \frac{\Delta A}{A_0}\Delta\varepsilon(t), \tag{3}$$

with $\Delta\varepsilon(t)$ having the form

$$\Delta\varepsilon(t) = \Delta\varepsilon e^{-t/\tau} \tag{4}$$

for the simplest case of a system with a single relaxation timescale τ.

A similar terminology as above applies to shear deformations, whose response is in general determined by the shear modulus $G(t)$.

2.5. The method of probing compression and shear simultaneously through a pressure relaxation

Applying a step strain and then measuring the stress relaxation over time is one of the classical experiments to characterize the viscoelasticity of a material. In the case of a surface monolayer, the surface area can be quickly changed by moving the surface barriers, and the surface pressure is observed over time. In the experiments reported here, surface area is changed (reduced) by 10%, and the pressure is observed for 30 min. Then the layer is compressed by another 10%. Fig. 3 shows this compression history.

At equilibrium, the pressure tensor of a simple fluid is isotropic and involves only the scalar hydrostatic pressure. This means that the surface pressure measured by the Wilhelmy plate method is independent on the orientation of the plate itself. However, the anisotropic compression that is performed by moving barriers in the typical rectangular Langmuir trough (see Fig. 1) takes the system into a non-equilibrium configuration, in which the pressure tensor is initially anisotropic and then relaxes towards equilibrium. The perpendicular pressures will relax to the same value provided that the system is a viscoelastic liquid, i.e., that over a sufficiently long timescale the shear stress decays to zero. This Langmuir trough experiment is conceptually equivalent to what can be done in computer simulations, for examples see the molecular dynamics simulation of stress relaxation for both a Lennard-Jones and a soft sphere fluid [25] and the Brownian dynamics simulations in [26].

This idea of measuring the surface pressure anisotropy, as a way to probe both the compression and shear moduli of a surface film, was recently proven in [11]. In that previous work, oscillatory deformations were applied. The technique was also compared to other existing surface rheometry methods, in particular [12,27]. The main advantage of the pressure anisotropy method lies in its simplicity and economy. Only standard apparatus available in any surface science laboratory (the Langmuir trough and Wilhelmy plate tensiometer) are required. There have been attempts to design Langmuir troughs with compression geometries that avoid shearing the monolayer. These are either circular or rhombic troughs [28,29]. If the compression of a viscoelastic monolayer is done in one of those geometries, the surface stress tensor will remain isotropic. Using uniaxial compression in a rectangular trough, and measuring the pressure in orthogonal directions, is a way to turn a potential problem into a way to extract useful information. Without repeating the full derivation [11,30] we simply recall that it is possible to relate the principal components of the surface stress tensor to the applied uniaxial strain in the x-direction, starting from the general expressions for a homogeneous viscoelastic medium and within the limits of a linear viscoelastic response [30]. In the case of a fast compression, the pressure in the directions parallel and perpendicular to the compression direction would be:

$$\begin{aligned}\Pi_{xx}(t) &= \Pi_0 + \frac{\Delta A}{A_0}\big(\Delta\varepsilon(t) + \Delta G(t)\big),\\ \Pi_{yy}(t) &= \Pi_0 + \frac{\Delta A}{A_0}\big(\Delta\varepsilon(t) - \Delta G(t)\big).\end{aligned} \tag{5}$$

2.6. A comment on aging in protein films

For systems that have slow dynamics, choosing an appropriate experimental protocol is necessarily a balance between what would be ideal—equilibrium starting conditions and infinitely long relaxations—and what is experimentally practical. Our compression protocol (Fig. 3) has the advantage that the full concentration range from quite dilute (near the overlap concentration for isolated protein coils) up to the highest concentrations possible for the protein monolayer is covered in a reasonable time. The disadvantage is that the monolayer history is complex, i.e., we do not make a new spread monolayer for each compression. There are two underlying assumptions: (1) that the monolayer will have equilibrated before the next compression, and (2) that the monolayer age over a day is less important than the effect of concentration. As a control experiment to test the effect of not waiting for complete relaxation before a new compression, single relaxation experiments were performed in which the pressure was recorded for many hours. The relaxations were found to be the same. The second of these conditions, aging, is the most delicate, and scientifically interesting as well. There is considerable literature showing strong effects of age on the properties of protein layers [23]. These experiments are on adsorbed layers, and show changes over timescales between one and several hours. Some of this time-evolution of surface properties might be linked to the increase of material on the surface. In other cases it has been attributed to the internal restructuring of the layer, with protein unfolding and formation of stable covalent bonds. Aging has not been investigated systematically here, it will be the focus of future work. Preliminary data (not shown) indicates that aging in spread layers is less significant than in adsorbed layers. This may be due to the fact that spread layers (in the concentration range explored here) are single-molecule thick films as opposed to the multilayer structure of adsorbed films. Confinement to a monolayer means that the protein has less freedom to restructure its conformation and therefore also less chance to form inter-protein bonds.

3. Results and discussion

3.1. Equilibrium data

Fig. 2 shows the equilibrated (steady state) pressure isotherms data for β-casein and β-lactoglobulin, under the two pH conditions of this investigation. The low pH, 6.0, is quite close to the isoelectric point of both proteins (around 5.5), where there is no net charge on each protein. In this condition, as reported before [9,10], the protein chain is at its most compact and resembles a random walk. This is reflected in steep pressure isotherms, due to more compact chain conformations. At the higher pH, 8.3, there is sufficient net charge for the protein chains to have a much more expanded configuration. In analogy with polymers, this conformation can be thought of as a self-avoiding random walk. The surface pressure isotherm is less steep. In all cases, for the pressure region between around 0.5 and 5 mN/m, pressure increases like a power of the surface concentration, and the power law scaling exponent can be recovered from the isotherm. This semi-dilute regime was explored in detail in previous work [9,10]. The scaling exponents for these systems are known from this previous work, and they quantify the average swelling of the molecular conformation: 5.3 for β-lactoglobulin pH 8.3, 8.3 for β-lactoglobulin pH 6.0, 4.8 for β-casein pH 8.3, 6.7 for β-casein pH 6.0.

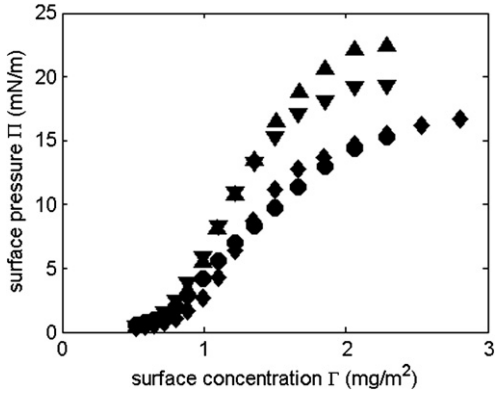

Fig. 2. Equilibrium surface pressure as a function of the surface concentration for the four systems studied in this work: β-lactoglobulin pH 8.3 (▼) and pH 6.0 (▲), and β-casein pH 8.3 (●) and pH 6.0 (◆).

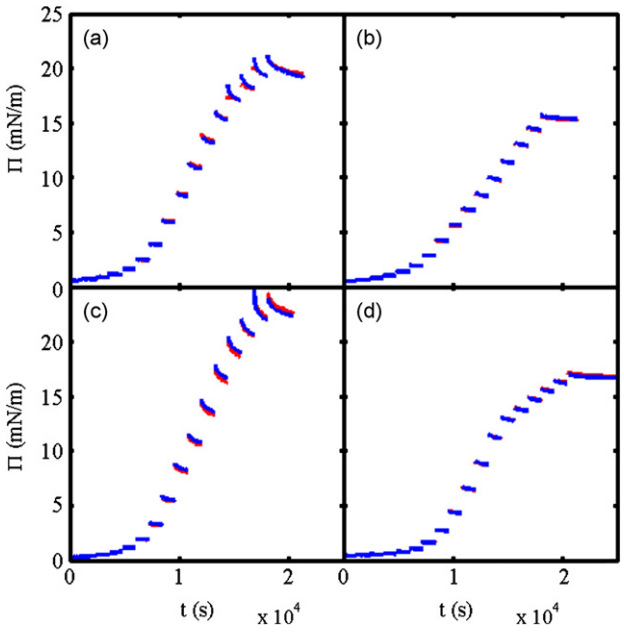

Fig. 3. Pressure data collected by the two perpendicular sensors as a function of time. The difference between the measurements from perpendicular sensors is noticeable in panels (a) and (c), corresponding to β-lactoglobulin pH 8.3 and β-lactoglobulin pH 6.0, respectively. The higher pressure is Π_{xx}. In panels (b) and (d), β-casein pH 8.3 and pH 6.0, the two surface pressures are indistinguishable throughout the experiment. Fig. 4 shows how these signals are analysed in terms of compression and shear relaxations.

3.2. Relaxation data: compression modulus

Fig. 3 shows the experimental data collected by performing fast (∼30 s) compressions of the monolayer films.

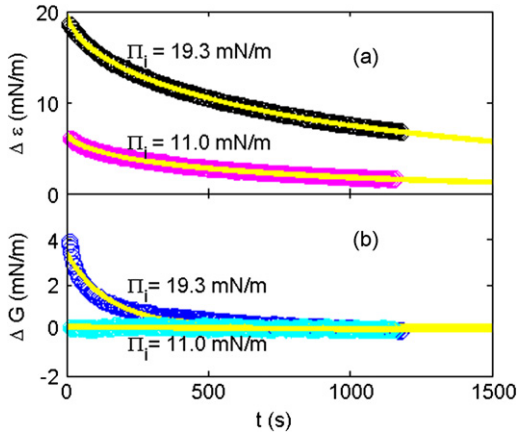

Fig. 4. (a) shows typical values of the average of the signals from perpendicular pressure sensors. This is data from β-lactoglobulin pH 8.3, at the pressures indicated on the figure. This average signal is the compression modulus relaxation. (b) shows the difference between the surface pressures Π_{xx} and Π_{yy} (see Fig. 1), from the same raw data as panel (a). This difference signal is the shear modulus relaxation. In both panels, the light line through the data points is a fit as described in the text.

This kind of step-compression experiment for monolayers is reported by Monroy and co-workers [13,14,31,32] in a series of papers on various polymer systems, by Wustneck et al. [33] on sodium eicosanyl sulfate monolayers, and by Rodriguez Nino et al. [34] for β-casein.

Our recent understanding of the surface pressure anisotropy shows that in the presence of a finite shear modulus these measurements should be performed by measuring surface pressure in both directions [11]. Otherwise the contribution from shear cannot be separated from the (main) effect of compression modulus relaxation, and extracting amplitudes and timescales from the mixed measurement may not be meaningful. Extracting a spectrum of timescales is especially misleading if a single sensor is used, as the decay will arise out of a combination of two independent relaxation processes.

By following the approach in Eq. (5), i.e., looking at the average and the difference of the perpendicular pressure values, both compression and shear relaxations can be identified. These are plotted in Fig. 4 for a few selected cases. A condition where the shear modulus relaxation is particularly prominent is shown in Fig. 4b.

Even after removing the contribution from the shear modulus, we found that the compression modulus relaxation was still a complex decay, that could not be fitted with a single exponential decay. This is proved in Fig. 5c, showing the result of fitting compression decay with the functional form

$$\Delta\varepsilon(t) = \Delta\varepsilon \cdot e^{-(t/\tau)^{\beta}} \tag{6}$$

with β allowed to optimize. β would be equal to 1 if a single relaxation timescale was present in the system, and a value of $\beta < 1$ is indicative of a spectrum of relaxation times and is often found in complex systems such as gels [35]. The averages of β values for these systems are 0.59 ± 0.13 for β-lactoglobulin pH 8.3 (▼) and 0.50 ± 0.13 for pH 6.0 (▲), and 0.60 ± 0.07 for β-casein pH 8.3 (●) and 0.66 ± 0.05 for pH 6.0 (◆). All the values for β are clustered around 0.6, so assuming this reflects some common underlying physical process this value was fixed for all the data sets.

Figs. 5a and 5b show the result of fitting the compression modulus relaxation with the stretched exponential form with β fixed to 0.6. This decay form indicates that there is quite a wide set of decay times, with τ being an average timescale. The amplitude of the relaxation increases with surface concentration and with the surface pressure. This is not surprising, since the equilibrium compressional modulus is also growing proportionally to the pressure. What is more surprising is the behavior of the decay timescales with increasing pressure in the various systems. β-Lactoglobulin at both pH values and β-casein at high pH exhibit slow relaxations, with τ increasing from about 200 s at low pressure up to around 600 s at high pressure. β-Casein at low pH shows much slower relaxations, on the order of 30 min. We do not have an explanation for this striking difference, other than pointing to the fact that the molecular conformation in this low pH condition is quite compact. As a general remark, we note that only a physical state such as a gel or glass can account for timescales which are so different from those of molecular or hydrodynamic diffusive motion.

Complex, non-single exponential decays are quite commonly found, both in polymer and small molecule monolayers. Sometimes similar data is fitted with a sum of 2 exponential decays, as in [34] and also our own previous work [9]. Although these functions also give a very good fit of the data, there is no reason to expect exactly 2 timescales, and the 2 timescale function has 5 free parameters instead of the 3 parameters required by the analysis presented here.

It should be noted that due to temperature fluctuations, subphase evaporation and mechanical instability of the pressure sensor, it is very difficult in this experiment to measure relaxations reliably on timescales longer than a few hours. This is unfortunate, because a test of the decay form Eq. (6) is that the pressure should reach a plateau when plotted against the logarithm of time but, given the fitted parameters the plateau should appear after around 3 h.

3.3. Relaxation data: shear modulus

Figs. 6a and 6b show the result of fitting the shear modulus decay with a simple exponential form. This simple decay form was chosen because the shear modulus is a relatively small signal, and its noise was such that we could not reliably determine a good stretch exponential factor. However the imperfect fit of the data in Fig. 4b at $\Pi = 19.3$ mN/m does indicate that very likely this decay has a collection of timescales, as for the case of the compression modulus.

Fig. 6 shows only data from β-lactoglobulin, because β-casein resulted in a negligible decay throughout the range of surface conditions explored here. Even for β-lactoglobulin the shear modulus is negligible, until the pressure of around 12 mN/m. From that threshold onwards there is a close to linear increase. This similar trend was identified in two previous papers were β-lactoglobulin was investigated with different methods [11,12]. Fig. 6b appears to show a timescale increasing to a few minutes and even tens of minutes. At present the

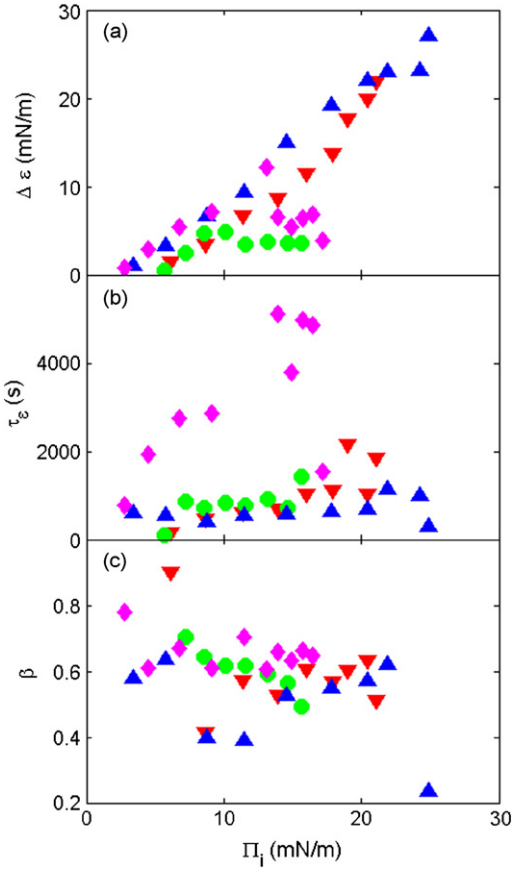

Fig. 5. The amplitude (a) and timescale (b) of the compression modulus relaxation, fitted with a stretched exponential function with stretch exponent set to 0.6. These parameters are plotted as a function of the pressure at the start of the relaxation. Panel (c) shows the values of the stretch exponent if this is allowed to float. Symbols correspond to the four systems investigated: β-lactoglobulin pH 8.3 (▼) and 6.0 (▲), and β-casein pH 8.3 (●) and 6.0 (◆).

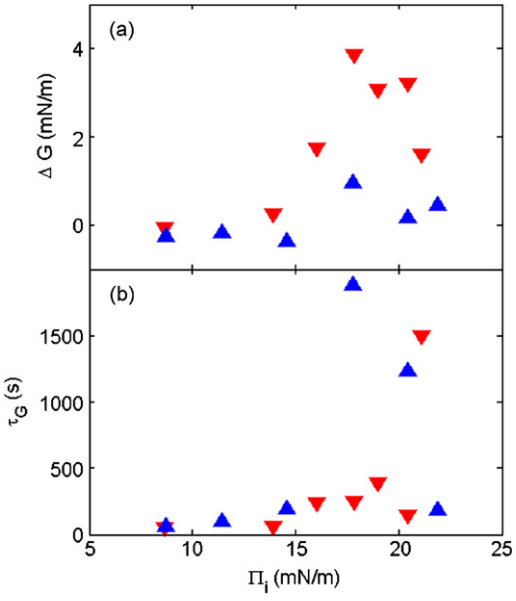

Fig. 6. The amplitude (a) and timescale (b) of the shear modulus relaxation, obtained by fitting the relaxation with a simple exponential decay function. Only β-lactoglobulin pH 8.3 (▼) and pH 6.0 (▲) are shown since for β-casein this shear relaxation is negligible throughout the investigated range.

data is too little to speculate on precise trends in the timescale. It is interesting to note that there is no clear model available to understand the origin of these extremely long timescales.

3.4. Fluid and soft solid surface layers

A finite shear elastic modulus is in principle an indication of a chemical gel formed by attractive inter-protein interactions or a sterically jammed state, where the interactions are purely repulsive and dynamics are hindered by excluded volume. For β-lactoglobulin, the evidence points towards the fact that the surface layer is a physically jammed state for surface pressures above ∼10 mN/m. This is supported by the similarity of viscoelastic data between 2D hard colloids and β-lactoglobulin layers [12], by the fact that the threshold concentration for emergence of a shear modulus is so high [11], and finally by very recent experiments where the presence or absence of chemically active groups was shown not to play an important role [36].

It is very interesting that in contrast to β-lactoglobulin layers, β-casein layers remain fluid at all concentrations. In previous work we investigated the surface conformation of β-casein [9]. As the surface concentration is increased, β-casein undergoes a transition from a state where all the molecule lies on the surface to desorbing a tail of the chain into the subphase. This picture has been proposed early on based on the shape of the adsorption curve [15,16], and evidence in favour of it continue to be presented [37]. We believe that this transition is what enables β-casein molecules to remain mobile in concentrated surface layers. Above the tail-formation surface pressure, and in contrast to β-lactoglobulin, β-casein molecules have the freedom to crawl over/under their neighbours.

4. Conclusions

We have reported measurements of pressure relaxation following a step-strain in a wide range of surface concentrations, for two proteins each at two subphase conditions. In contrast to previous similar experiments, we have measured the surface pressure anisotropy during the relaxation, and this has enabled us to identify separately the contribution from the compressional and the shear moduli, in a single experiment. We have made measurements as a function of surface concentration. Both moduli exhibit long-time relaxations that are poorly understood. Even after identifying the contribution from each modulus (a problem that may very well be present in all previous literature dealing with similar experiments), the relaxation dynamics remain non-trivial. The simplest form that describes these relaxations is the stretched exponential decay. This form is commonly seen in gels and glassy materials and indicates that a range of relaxation timescales are active in the system. For the same step-strain, the amplitude of the compressional modulus relaxation is much larger for β-lactoglobulin than for β-casein, and especially above the pressure of the β-casein conformation transition [9]. This indicates that the β-casein tail submersion has a strong effect on reducing the stresses that can be stored in the monolayer, presumably by increasing the mobility of the

β-casein molecule. Regarding the shear modulus, it is found to be negligible for β-casein, and to have finite values in β-lactoglobulin layers only above a threshold concentration. We have argued that this is consistent with a physically repulsive jammed state for β-lactoglobulin at high surface concentration.

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