Comparative Study of Adsorbed and Spread β-Casein Monolayers at the Water−Air Interface with the Pendant Drop Technique

J. Maldonado-Valderrama, H. A. Wege, M. A. Rodríguez-Valverde, M. J. Gálvez-Ruiz, and M. A. Cabrerizo-Vílchez*

Biocolloid and Fluid Physics Group, Department of Applied Physics, University of Granada, E-18071 Granada, Spain

Received February 12, 2003. In Final Form: July 4, 2003

A Langmuir-type pendant drop film balance has been applied in the study of protein monolayers at the water−air interface in a comparative study of adsorbed and spread β-casein layers. Trurnit's method for spreading proteins at the air−water interface has been modified and thoroughly adapted to the requirements of the pendant drop technique. Adsorbed β-casein layers have been obtained by means of double coaxial capillary that enables a subphase exchange, once the desired amount of protein attains the interface. Precisely, this becomes one of the most practical aspects of this technique, allowing a direct comparison between spread and adsorbed layers. Moreover, it provides an alternative to conventional spreading methods for obtaining protein layers and promises to be specially useful in the study of globular protein monolayers, which are very difficult to obtain with conventional methods. Comparison between spread and adsorbed $\pi-A$ isotherms and static elasticity offers an excellent concordance, which increases in successive compression−expansion cycles, suggesting that a similar structural configuration is finally adopted at the interface independently of the technique (spreading or adsorption) used to form the layer.

Introduction

Many functional properties of proteins derive from the structure they adapt at interfaces. For this reason, the question about protein interfacial conformation has widespread relevance. The knowledge of protein layer composition and conformation is essential in many fields, e.g., in the food industry for emulsification and foaming, in which proteins are used as stabilizers.[1,2] The conformational state of a protein located at an interface differs from that of a bulk protein since their immediate environment is different. According to the specific properties of the interface,[3−5] proteins undergo a structural transformation called surface denaturation. Whether this modification is reversible or not has been widely studied in the literature[6,7] and although it has been shown to be irreversible under several experimental conditions, the question still remains open.

The starting point of the present work is a reversibility analysis of protein adsorption at the water−air interface using a pendant drop surface balance equipped with a rapid subphase-exchange device.[8,9] In these experiments, after adsorption from bulk solution onto the interface, the subphase is exchanged and the desorption process is studied, as done before by other authors using conventional surface balances.[7]

There are two common ways to obtain protein layers at the liquid−air interface. One is by adsorption from bulk solution: the layer is formed by diffusion of dissolved protein molecules onto the interface, followed by molecular rearrangement.[7,10] This has been widely studied with conventional balances,[7] as well as on the pendant drop technique.[11] The other is by spreading the protein from a spreading solution, with several methods existing that provide interfacial films.

A main difficulty of spreading proteins resides in the fact that in their native state many of them present some solubility in the aqueous subphase, which is too high to form stable monolayers. A good way to overcome this is to denaturate them prior to the deposition onto the interface. For this study, Trurnit's method for spreading proteins[12] was adapted to the requirements of the pendant drop technique−mainly due to the small dimensions of interface−so that monolayers of spread β-casein could be formed. Surface pressure−interfacial area isotherms of monolayers are generated by changing the drop volume in a controlled manner and simultaneously measuring surface tension and interfacial area, which provides information about structural and morphological characteristics of spread monolayers.[13−15]

The main practical advantage of the technique exposed lies in the possibility of obtaining adsorbed protein

(1) Rodríguez Patino, J. M.; Rodríguez Niño, M. R. *Colloids Surf., B* **1999**, *15*, 235.
(2) Dickinson, E. *Colloids Surf.* **1989**, *42*, 191.
(3) Green R. J.; Hopkinson, I.; Jones R. A. L. *Langmuir* **1999**, *15*, 5102.
(4) Segumpta, T.; Razumovsy, L.; Damodaran, S. *Langmuir* **1999**, *15*, 6991.
(5) Beverung, C. J.; Radke, C. J.; Blanch, H. W. *Biophys. Chem.* **1999**, *81*, 59.
(6) MacRitchie, F. In *Proteins at Liquid Interfaces*; Moebius, D., Miller, R., Eds.; Elsevier: Amsterdam, 1998; p 149.
(7) Graham, D. E.; Phillips, M. C. *J. Colloid Interface Sci.* **1979**, *70*, 403.
(8) Cabrerizo Vilchez, M. A.; Wege, H. A.; Holgado-Terriza, J. A. *Rev. Sci. Instrum.* **1999**, *70*, 2438.
(9) Wege, H. A.; Holgado-Terriza, J. A.; Cabrerizo Vilchez, M. A. *J. Colloid Interface Sci.* **2002**, *249*, 263.
(10) Tripp, B. C.; Magda, J. J.; Andrade, J. D. *J. Colloid Interface Sci.* **1995**, *173*, 16.
(11) Makievski, A. V.; Fainerman, V. B.; Bree, M.; Wüsteck, R.; Krägel, J.; Miller, R. *J. Phys. Chem. B* **1999**, *102*, 417.
(12) Trurnit, H. J. *J. Colloid Interface. Sci.* **1960**, *15*, 1.
(13) Rodríguez Niño, R.; Carrera, C. S.; Rodríguez Patino, J. M. *Colloids Surf., B* **1999**, *154*, 343.
(14) Krägel, J.; Grigoriev, D. O.; Makievski, A. V.; Miller, R.; Fainerman, V. B.; Wilde, P. J.; Wüsteck, R. *Colloids Surf., B* **1999**, *12*, 391.
(15) Min, D. J.; Winterton, L. Andrade, J. D. *J. Colloid Interface Sci.* **1998**, *197*, 43.

10.1021/la034242z CCC: $25.00
Published on Web 09/03/2003

monolayers likely to be compared to the spread ones, since, to compare adsorbed and spread protein behavior at interfaces, similar conditions must be accomplished. To be precise, in the process of formation of layers by adsorption, protein remaining in bulk after adsorption may lead to formation of multilayers and adsorption of new molecules on to the interface upon expansion, whereas the amount of protein in spread layers remains constant, and its compression−expansion cycles enlighten configurational changes at the interface as well as formation of bidimensional structures. An appropriate subphase exchange provides an adsorbed protein layer that is, finally, suitable for a reliable comparison with a spread protein layer. There are some attempts in the literature to compare behavior of spread and adsorbed protein layers;[6,14,16] nevertheless, these are done in conventional surface balance, and no direct comparison between isotherms is shown.

In the present work, a comparison between spread and adsorbed β-casein layers with the pendant drop technique is presented in terms of their isotherms and static elasticity, and a novel alternative to conventional deposition of monolayers of soluble surfactants is proposed.

Materials and Methods

Protein and Buffer Solutions. Lyophilized, essentially salt free bovine milk β-casein (90+% by electrophoresis) was purchased from Sigma Chemical Co. It is made up of 209 amino acids, and it has a molecular weight of 23 800 Da; the entire molecule has an average hydrophobicity of 5.58 kJ/residue.[10] It was stored at −18 °C and used without further purification. The aqueous subphase used in this study was a phosphate buffer saline (PBS): 13.0 mM KH_2PO_4 (purissimum, Merck), 100 mM NaCl (p.a., Merck), 54.0 mM Na_2HPO_4 (p.a., Merck) of pH 7.4. To prevent bacterial contamination, 0.5 g/L NaN_3 were added to the buffered solvent. Milli-Q purified water (0.054 mS) was used for buffer preparation and all other purposes. Only freshly prepared solutions were used. All experiments were performed at $T = 23$ °C, and all solid surfaces in contact with the drop, the bulk phases, or the spreading solvent were made of glass, Teflon, or stainless steel. Prior to each experiment, all the glassware was cleaned in hot chromic sulfuric acid and then repeatedly rinsed with water. Solutions were filtered with a 0.2 μm PFTE filter. Before each experiment, the pendant drop alimentation tubes were flushed with protein solution, to minimize the loss of protein by adsorption onto the tubes. The surface tension γ_0 of the clean interface was measured before each experiment to ensure the absence of surface active contaminants in the solution. The reproducibility of the experiments was verified through replicate measurements.

Setup. The experiments were performed with a constant surface pressure penetration Langmuir balance based on axisymmetric drop shape analysis (ADSA), which is described in detail elsewhere.[9]

The whole setup, including the image capturing, the microinjector, the ADSA algorithm, and the fuzzy pressure control, is managed by a Windows integrated program (DINATEN). It is represented in Figure 1. A solution droplet is formed at the tip of the coaxial double capillary, connected to a double microinjector. The program fits experimental drop profiles, extracted from digital drop micrographs, to the Young−Laplace equation of capillarity by using ADSA, and provides as outputs the drop volume V, the interfacial tension γ, and the surface area A. Pressure and area control use a modulated fuzzy logic PID algorithm (proportional, integral, and derivative control). They are controlled by changing the volume. The isotherms are generated by changing the drop volume in a controlled manner and simultaneously measuring surface tension and surface area. The surface pressure values are obtained from the relationship $\pi \equiv \gamma_0 - \gamma$, where π is the surface pressure, γ_0 is the surface tension of pure liquid, and γ is the surface tension of the liquid covered with the monolayer.

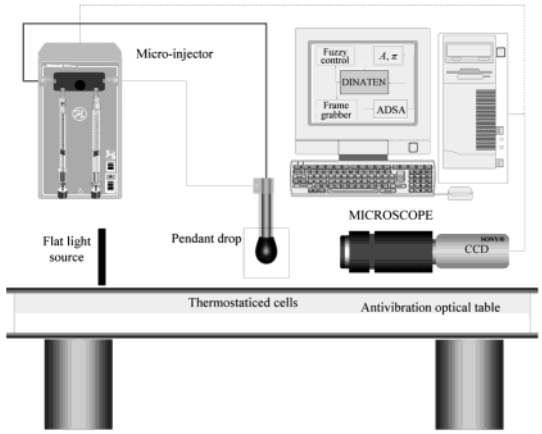

Figure 1. Setup.

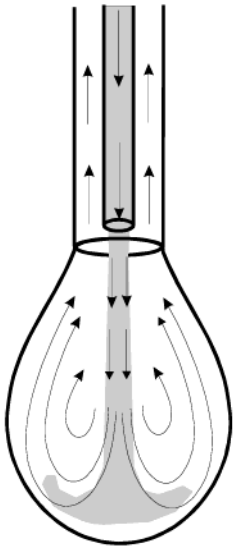

Figure 2. Scheme of the subphase exchange process.

Adsorbed β-Casein Layers. Studies of protein desorption were performed as follows: a protein solution drop is formed at the tip of the capillary and kept at constant surface area while the protein adsorbs freely at the interface, forming a layer. Once the desired surface pressure is attained, the bulk solution in the drop is substituted by the aqueous subphase. This is done by extracting simultaneously the bulk solution through the outer capillary and injecting through the inner one the same amount of aqueous subphase at the same rate.

Under the chosen conditions, this process is completed after 36 s. A schematic diagram of this process is shown in Figure 2. After the exchange, the protein layer is studied under different experimental conditions. First, the behavior at constant surface area is analyzed. Once equilibrium is reached after the exchange, i.e., when no more significant variation of π is recorded, the stability of the layer is evaluated by expanding the drop to the maximum area (50 mm^2) and repeatedly compressed and expanded: only when $\pi(A)$ is a single-valued function for all compression−expansion cycles is the layer considered stable, and the data are used for the isotherms.

Spread Monolayers. The deposition of the monolayer is one of the most critical points of this technique. Several methods for spreading monolayers[17] have been tested, but all resulted in incomplete spreading of the protein, except the adaptation of Trurnit's method,[12] which finally provided satisfactory results when compared with the findings of other authors (not shown). This method consists of allowing an aqueous protein solution to flow down a glass rod before contacting the water surface, and

(16) Gau, C. S.; Yu, H.; Zografi, G. *J. Colloid Interface. Sci.* **1994**, *162*, 214.

(17) MacRitchie, F. *J. Colloid Interface Sci.* **1986**, *25*, 341.

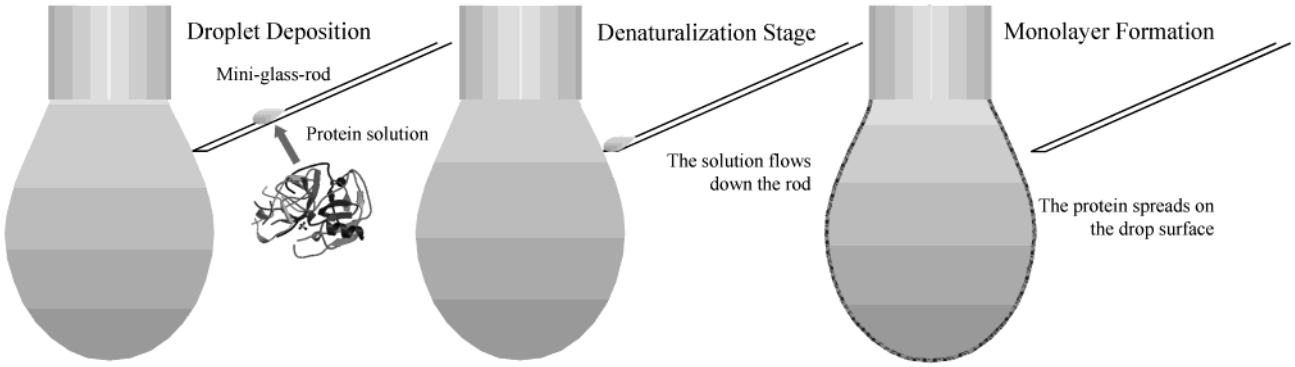

Figure 3. Stages of the deposition method, based on an adaptation of Trurnit's method for spreading proteins.

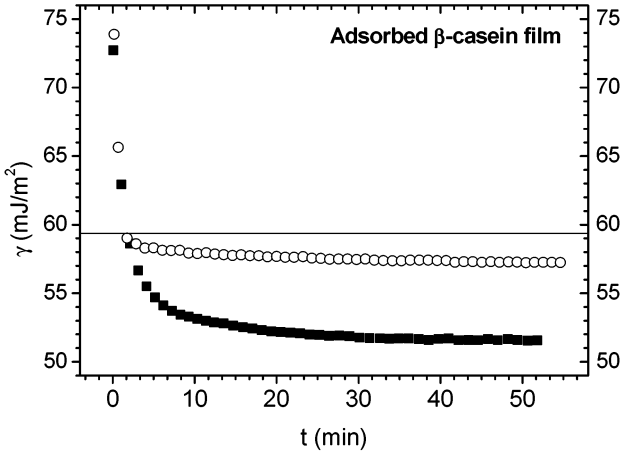

Figure 4. Typical result of a subphase exchange of a β-casein drop (0.03 g/L in PBS, pH 7.4, $T = 23$ °C): solid squares, surface tension of a solution drop; open circles, subphase exchange at surface pressure equal to 10 mJ/m^2.

has been used on conventional film balances by a number of workers.[13,15] In this study, a "mini glass rod", a 0.3 mm diameter glass cylinder, was used on which a known volume (1 μL) of protein solution was placed. This droplet was allowed to flow along the rod and then brought into contact with the drop surface. Finally the denaturated protein spreads on the drop surface.

A schematic diagram of the method is shown in Figure 3. To ensure a complete spreading, 30 min were allowed to elapse before $\pi-A$ isotherms were recorded. The compression rate for β-casein was 0.27 mm^2/s, a value at which best reproducibility was attained and only a slight hysteresis was found on the expansion−compression cycles. With a "soft" protein like β-casein,[10,15] no special precautions for spreading have to be adopted, and purely aqueous solutions of protein are satisfactory for spreading monolayers. Thus, this method avoided the contamination problems derived from the use of other spreading solvents.

Results and Discussion

Preliminary Results of Reversibility of Protein Adsorption. The reversibility of β-casein adsorption at the water−air interface was tested in experiments as described above. After subphase exchange at a certain surface pressure, the behavior of the adsorbed protein was studied by monitoring γ at constant A: an increase in γ would indicate desorption of molecules from the adsorbed layer. A typical result of such an experiment for $\pi_{exchange} = 10$ mJ/m^2 is shown in Figure 4.

After the exchange, the surface tension not only does not increase, but it even decreases. This suggests that under the chosen conditions, adsorption is irreversible. The observed decrement in $\gamma(t)$ could be due to further unfolding of the already adsorbed molecules, but also to further adsorption of molecules remaining in the bulk after the subphase exchange. For this reason, i.e., in order to avoid any possible inaccuracy as a result of an incomplete subphase exchange, a detailed analysis of the efficiency of the subphase exchange was performed.

Efficiency of the Subphase Exchange. The efficiency of the subphase exchange has been checked previously using different pure liquids.[8] However, since the situation is somewhat different for surfactant solutions, the following test series was performed: solution drops were generated, and after a short equilibration time the subphase was exchanged, varying inherent exchange parameters such as flow direction, injection speed, and the exchanged volume. The $\gamma(t)$ evolution of these "exchanged drops" was used to evaluate the efficacy of the subphase exchange: it was considered to be complete if the surface tension of the exchanged drop remained constant in time.

The effect of the volume and through-flow speed is analyzed in Figure 5 for a solution drop of β-casein, 0.03 g/L. For comparison, a reference curve for no exchange, i.e., a $\gamma(t)$ curve for a unchanged protein solution, was included. It can be seen that both parameters are interdependent and that the efficiency of the exchange depends mainly on the through-flow velocity. At low speeds the exchange does not appear to be complete. The injected liquid probably does not reach the interface, and some protein solution remains in the subsurface after the exchange. The optimum value for the through-flow velocity seems to be 10 μL/s, as there is no appreciable change in surface tension after the exchange. The effect of the exchanged volume depends on the through-flow velocity: an increase in the volume does not seem to improve the exchange at low speeds. Note that an exchange of 200% of the drop volume at 10 μL/s is not satisfactory, protein remains in the bulk, and surface tension decreases with time, while the original subphase seems to be completely removed and substituted with an exchange of 400% of the drop volume at the adequate velocity, in agreement with previous experiences.[9]

Effect of Adsorption Pressure on Adsorbed β-Casein Films. Figure 6 shows the $\gamma(t)$ data before and after subphase exchange from several experiments with 0.03 g/L β-casein that differ by the exchange pressure π_{ex}. A reference curve without exchange is included. For all π_{ex}, the γ values immediately before and after exchange coincide, indicating that the protein film endures the process at all pressures.

Also, for all cases we observe that after an initial transient stage, i.e., once the subphase is depleted of protein, the surface tension remains essentially constant. This feature clearly suggests that the protein is well-attached to the interface, accounting for the irreversibility of

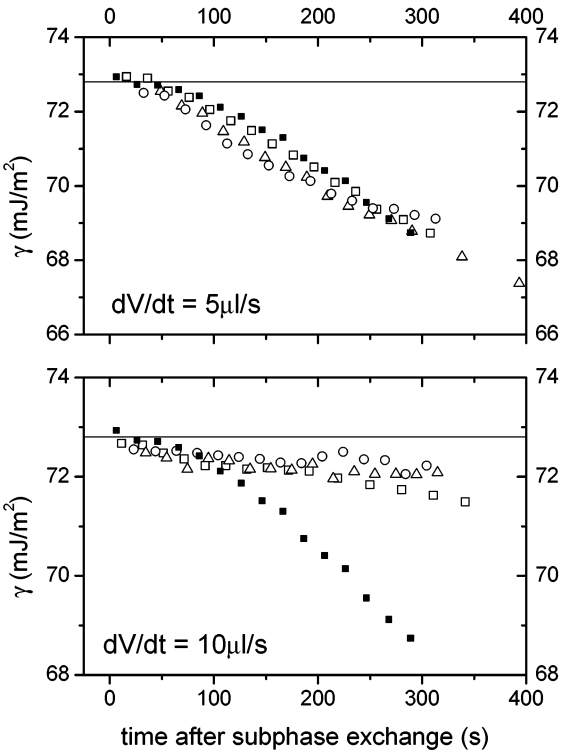

Figure 5. Experimental test of the subphase exchange. Effect of the exchanged volume and the through-flow velocity on the surface tension of a β-casein solution drop (0.03 g/L in PBS, pH 7.4, $T = 23$ °C) as a function of time: solid symbol, no subphase exchange. Exchange rate of 5 (upper) and 10 μL/s (lower): squares, 200%; circles, 400%; triangles, 600% volume exchanged.

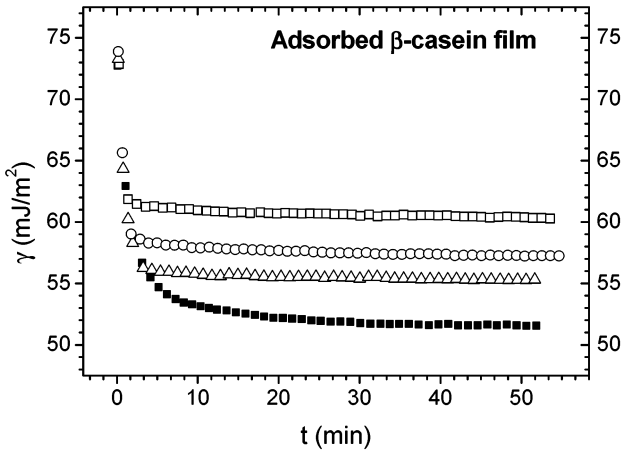

Figure 6. Effect of the subphase exchange on the surface tension as a function of time of a β-casein solution drop (0.03 g/L in PBS, pH 7.4, $T = 23$ °C) at different surface tensions measured at a constant surface area of 34 mm^2: solid squares, no subphase exchange; open symbols, subphase exchange at (squares) $\pi = 5$ mJ/m^2, (circles) $\pi = 10$ mJ/m^2, and (triangles) $\pi = 15$ mJ/m^2.

β-casein adsorption at the water−air interface[6,7] at all surface pressures.

A closer examination of the curves shows the effect of the pressure at which free adsorption from bulk is interrupted by subphase exchange on the resulting films: a short interval of sharp decreasing surface tension is observed just after the exchange that diminishes in magnitude with increasing π_{ex}. Since the subphase exchange was performed under optimum conditions, this reduction of surface tension must be due to molecular

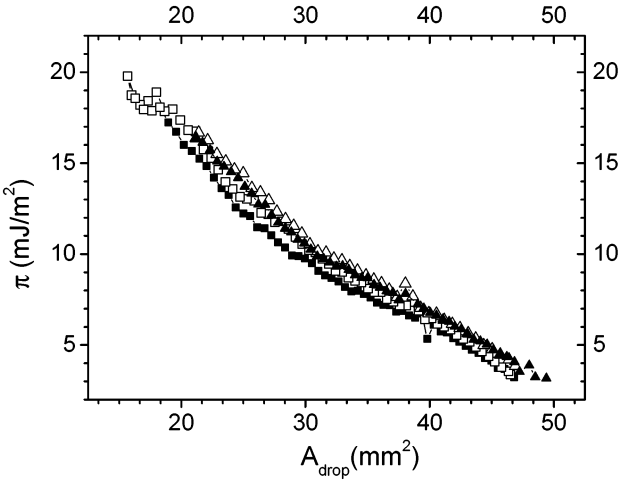

Figure 7. Reproducibility of the compression−expansion cycles for an adsorbed β-casein monolayer (0.03 g/L) obtained with an exchange at $\pi = 10$ mJ/m^2 for two different drops: squares, experiment 1; triangles, experiment 2; solid symbols, expansion; open symbols, compression.

rearrangement at the surface. This feature has also been found by other authors;[18−20] the degree of packing in the adsorbed layer seems to have a considerable influence on the degree of conformational change. That is to say, the lower the adsorbed amount of protein at the interface, the more the space the protein has to spread and unfold on the surface and, hence, to further reduce the surface tension.

Adsorbed Protein Monolayers at the Water−Air Interface. The stability of the adsorbed layers as deduced from Figure 6 suggests that they are suitable for compression−expansion isotherms, obtained by injecting and extracting clean buffer solution.

Figure 7 shows the reproducibility of the obtained isotherms, proving the reliability of the method. Behavior of β-casein layers was not found to be dependent on the surface pressure at which the subphase exchange was done (not shown) as long as the surface area at which it was performed provided zero pressure at the maximum area of drop; i.e., the higher the pressure, the smallest the drop. Moreover, different concentration solutions produced similar results in the $\pi-A$ isotherms (not shown). The parameters chosen for the experiments taking into account the dimensions of the drop, the high interfacial affinity of β-casein, and so the rapid increase of surface pressure upon adsorption on to the interface were subphase exchange at $\pi = 10$ mJ/ m^2 and an interfacial area of 27 mm^2. The $\pi-A$ isotherms (Figures 7 and 8) obtained under these conditions are represented versus the area of the drop given that, with this technique, we do not know the total amount of protein at the interface after adsorption from the bulk. Additional techniques will provide information of surface concentration and are under research.

Figure 8 shows the isotherms obtained upon two compression−expansion cycles. It is remarkable the coincidence between compression and expansion in the first cycle, suggesting that β-casein molecules adopt a partially unfolded structure which is perfectly reproducible upon compression−expansion of the layer. However, this coincidence seems to slightly diminish in the subsequent

(18) Miller, R. In *Proteins at Liquid Interfaces*; Mobius, D., Miller, R., Eds.; Elsevier: Amdsterdam, 1998.
(19) Dickinson, E. *Colloids Surf., B* **1999**, *15*, 161.
(20) Joos, P. *Biochim. Biophys. Acta* **1975**, *375*, 1.
(21) Gálvez-Ruiz, M. J.; Birdi, K. S. *Colloid Polym. Sci.* **1999**, *277*, 1083.

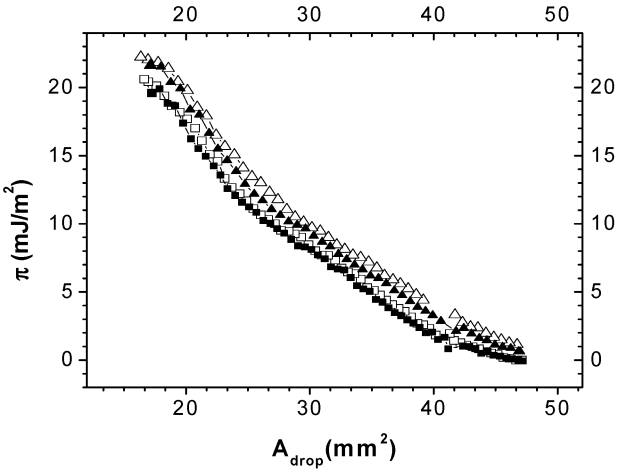

Figure 8. Effect of time on an adsorbed β-casein monolayer: squares, 10 min after subphase exchange; triangles, 50 min after subphase exchange; solid symbols, expansion; open symbols, compression.

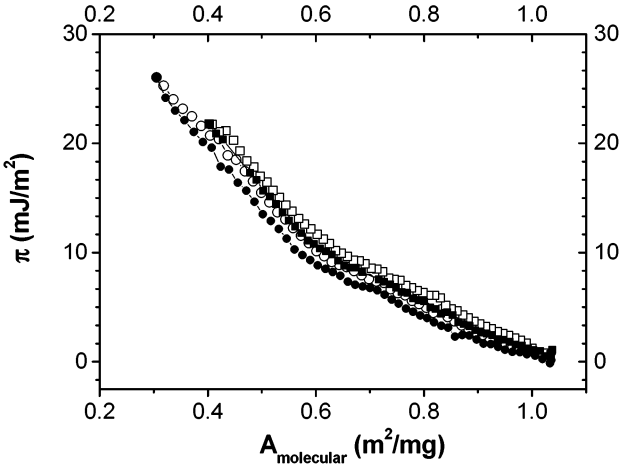

Figure 9. Effect of time on a spread β-casein monolayer: circles, 10 min after subphase exchange; squares, 50 min after subphase exchange; solid symbols, expansion; open symbols, compression.

cycle, performed after keeping the drop at the maximum area for 1 h. The structural configuration attained in this period (surface denaturation) is not as reproducible upon compression−expansion, and the subsequent compression−expansion cycle shows a slight hysteresis (Figure 8) and suggests that adsorption of casein onto the water−air interface is accompanied not only by a conformational change but possibly by an irreversible denaturation process.[14]

Spread β-Casein Layers at the Water−Air Interface. The efficiency of spreading by means of the adaptation of Trurnit's method was tested with the close agreement in replicate spreading of 1 μL of β-casein solution (0.048 g/L, which corresponds to a total amount of spread protein of 4.8×10^{-5} mg; not shown). The $\pi-A$ isotherms were plotted assuming that all protein molecules form the layer, and no loss due to desorption or diffusion into the bulk happens. The reproducibility of the isotherms and the slight hysteresis after continuous compression−expansion cycles, as can be seen in Figure 9, also guarantee a satisfactory spreading of the protein on the drop surface, and the monolayers can be considered insoluble once spread.

Moreover, the limiting molecular area, A_0, deduced by extrapolation of the straight portion of the plot $\pi-A$ to $\pi = 0$ [22] is $A_0 = 0.98 \pm 0.03$ m²/mg. This parameter is a measure of the interfacial area occupied by β-casein residues spread on the interface. It is known that proteins at interfaces spread to form monolayers that provide a limiting molecular area of around 1 m²/mg,[22] or else, $A_0 = 18.9$ Å²/residue. This value is comparable to the approximately 15−18 Å²/residue value obtained from X-ray diffraction data for various proteins.[21,22] The slight hysteresis found in the cycles (Figure 9) suggests that, as the protein is previously denaturated by means of the mini glass rod, it reaches the interface in a maximum unfolded state and is anchored by the maximum amount of trains of amino acids in contact with the interface. During compression, due to strongly increasing lateral interactions, the monolayer thickness increases according to the amount of loops and tails formed and a substantial expulsion of segments, and slow readjustment of loops and tails takes place. Upon expansion, the protein does not entirely recover the structural configuration attained in the spreading procedure, but preserves a partly unfolded structure responsible for the decrease observed in the hysteresis phenomena with the number of cycles (Figure 9). This feature has also been found by other authors,[23] suggesting that the protein slowly achieves a spontaneous unfolded structure at the interface.

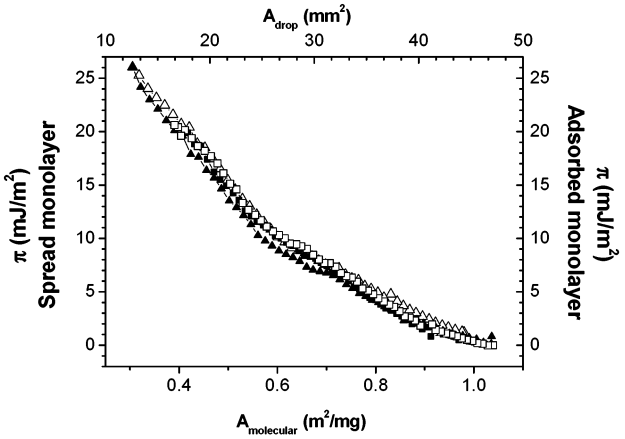

Figure 10. $\pi-A$ isotherms of the first compression−expansion: squares, adsorbed β-casein; triangles, spread β-casein; solid symbols, expansion; open symbols, compression.

Comparison between $\pi-A$ Isotherms for Adsorbed and Spread Layers. Experimental conditions have been achieved for a reliable comparison between spread and adsorbed monolayers. To compare their respective $\pi-A$ isotherms, the adsorbed isotherm has been shifted on the A-axis (Figure 10).

It is noteworthy the great similarity between adsorbed and spread isotherms which suggests that both methods lead to protein layers with comparable characteristics. However, the most interesting difference between adsorbed and spread isotherms is the different evolution of the hysteresis phenomena, as has been discussed in each section, respectively. Adsorbed β-casein layers exhibit less hysteresis than spread layers in the first cycle, and while this hysteresis diminishes with successive cycles for the spread layer (Figure 9), it seems to increase in the adsorbed layer (Figure 8). This feature might be due to the different ways that lead to a stable interfacial structural configuration[24] as has been explained above separately in each section.

(22) Birdi, K. S. In *Lipids and Biopolymer Monolayers at Liquid Interfaces*; Plenum Press: New York, 1989.
(23) Cornec, M.; Kim, D. A.; Narishmhan, G. *Food Hydrocolloids* **2001**, *15*, 303.
(24) Cho, D.; Cornec, M. A. *Bull. Korean Chem. Soc.* **1999**, *20*, 999.

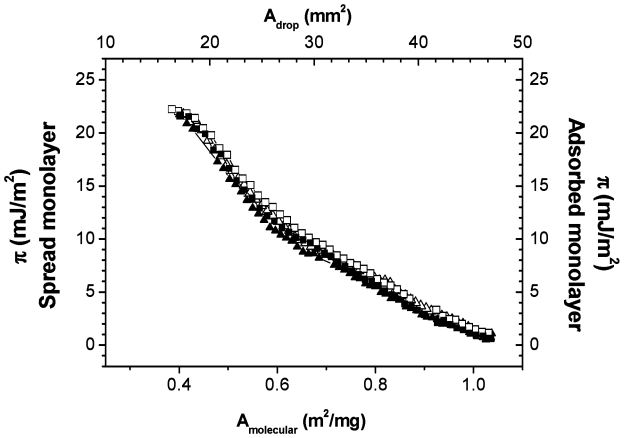

Figure 11. $\pi-A$ isotherms after 60 min at maximum area: squares, adsorbed β-casein; triangles, spread β-casein; solid symbols, expansion; open symbols, compression.

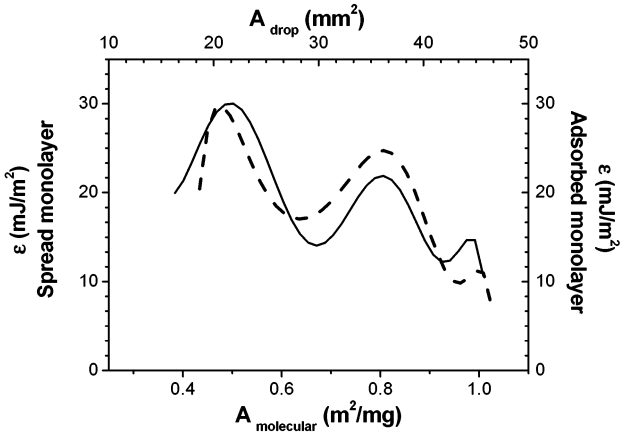

Figure 12. Elasticity modulus of β-casein monolayers in the first compression: solid line, spread layer; dotted line, adsorbed layer.

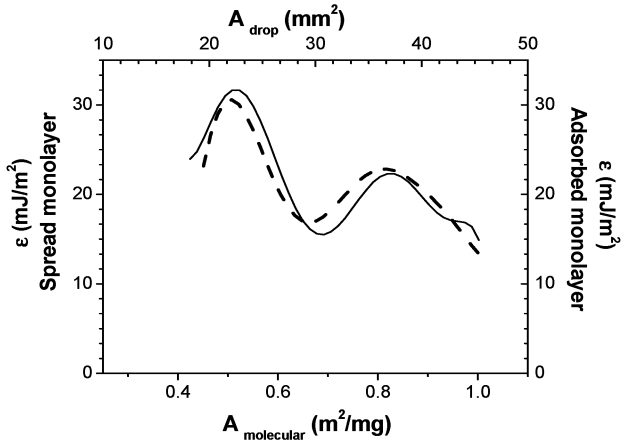

Figure 13. Elasticity modulus of β-casein monolayers after 60 min at the maximum area: solid line, spread layer; dotted line, adsorbed layer.

Briefly, spread β-casein is formerly unfolded, and upon leaving the protein 1 h at the maximum area, the protein does not completely retain such unfolded structure, but rearrangement of the protein chain leads to a configurational state in which the hysteresis phenomena has been reduced, as can be seen in Figure 9. Conversely, adsorption of β-casein onto the water−air interface is a spontaneous process, and the structure initially adopted might not be totally unfolded in the first instants. After leaving the adsorbed protein at the maximum area for 1 h, the protein is in a further unfolded structure that implicates a slight increase in the hysteresis phenomena upon new compression−expansion of the interface, as can be seen in Figure 8. Finally, Figure 11 shows the comparison of adsorbed and spread isotherms recorded after 1 h at the maximum area, and it can be seen that the concordance between the curves has improved from that the observed in Figure 10, not only in the shape of the isotherm but in the hysteresis phenomena.

***β*-Casein Monolayer Elasticity.** A closer examination of the curves also indicates critical surface pressure values at which the film properties change significantly through changes in the slope, as has also been noted by other authors for spread β-casein layers.[10,21] The static surface elasticity (ϵ) is expected to be equivalent to the dynamic surface elasticity extrapolated to zero[16] and is defined as $\epsilon \equiv -A(\mathrm{d}\pi/\mathrm{d}A)_T$. It is a measure of the film resistance to a change in area and can be easily calculated directly from the $\pi-A$ isotherms. The elasticity modulus provides information of the structure of the layer;[13,16] high elasticity values are associated with a film that has strong cohesive interfacial structure.

Anyway, structural changes of protein at interfaces are extremely complicated, and analysis of the elasticity modulus only does not provide a satisfactory conclusion. Figure 12 shows the variation of the elasticity modulus with the area of the layers. Although the shapes of the $\pi-A$ isotherms are almost identical for adsorbed and spread layers, the variation of the elasticity modulus is not as comparable. This feature might indicate different structural regions in the range of pressure studied characterized by an increasing elasticity modulus as the layer becomes more compressed. Similar results relating to differences in rheological properties of the protein layer that are not observed in the $\pi-A$ isotherms have also been found by other authors[14] and suggest that elasticity is particularly sensitive to structural changes. Taking into account the similar structure attained at the interface by spread and adsorbed molecules in view of their $\pi-A$ isotherms after successive compression−expansion cycles, it was interesting to analyze as well the evolution of the elasticity modulus.

Figure 13 shows the elasticity modulus of both layers in the final state attained. Differences between the elasticity modulus of both layers are substantially reduced, and this feature strongly accounts for a similar conformational state finally attained at the interface independently of the method used, as was previously deduced from the evolution of the isotherms.

Nevertheless, further analysis of the layer with other techniques is in progress so as to elucidate whether these changes in the slope correspond to different structures in the layer.

Conclusions

A new technique for comparing the behavior of adsorbed and spread protein at interfaces has been successfully developed on a Langmuir-type pendant drop film balance. Spread protein layers were obtained by a thorough adaptation of Trurnit's method[12] to the requirements of the pendant drop technique, obtaining a good reproducibility between different runs and barely hysteresis on the compression−expansion cycles. Adsorbed protein layers were obtained by means of a double coaxial capillary that enables a subphase exchange and provides an adsorbed protein film at the interface. No protein desorption from the interface was observed after the

exchange and upon compression and expansion of the layer, obtaining isotherms with scarcely hysteresis on the cycles.

$\pi-A$ isotherms of adsorbed and spread protein were shifted in A-axis and found to be fairly similar in the first compression−expansion cycle. Upon successive compression−expansion cycles, comparison of the isotherms shows even better coincidence in shape as well as in the hysteresis phenomena. Examination of the elasticity modulus of both layers shows the same analogy with increasing similarities with successive cycles. Taking into consideration these results, the structural configuration of protein layers finally attained at the interface appears analogous regardless of the method used in the formation of the layer.

The technique described for obtaining protein films by adsorption from bulk solution has clear advantages over conventional spreading methods: much less perturbation of the interface and minus diffusion into the bulk, absence of spreading solvents, no losses of protein remaining in the glass rod, and no external contamination of the interface. Furthermore, it promises to be especially useful in the study of globular proteins, which are very difficult to spread with conventional methods.[12] Besides, in view of the coincidence of adsorbed and spread isotherms, the comparison of the curves provides a reliable method for a quantification of the behavior of adsorbed protein monolayers upon compression and expansion of the interface.

Acknowledgment. Financial support from "Ministerio de Ciencia y Tecnología, plan nacional de Investigación científica, Desarrollo e Innovación Tecnológica (I+D+I)", Grants MAT2001-2834-C02-01 and AGL2001-3483-C02-02 are gratefully acknowledged.

LA034242Z