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Colloids and Surfaces B: Biointerfaces 19 (2000) 89–100

COLLOIDS AND SURFACES B

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β-Casein adsorption kinetics on air–water and oil–water interfaces studied by ellipsometry

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Accepted 8 December 1999

Abstract

In this work β-casein adsorption kinetics at air/water and oil/water surfaces is studied by ellipsometry. Double layer formation is observed; the process is not influenced by the type of the hydrophobic phase. A dense protein layer initially forms, and then the adsorption continues in a second more diffuse layer, which extends in the aqueous phase. The surface coverage (i.e. the adsorption, Γ) in the second layer approaches that in the first dense layer at long times, but the thickness and the refractive index of the second layer testify for lower volume density of protein there. The well known ellipsometric problem of correlating parameters in the case of measurement in a very thin layer (on a substrate which does not absorb light) has been overcome by using different upper phases (oil and air). This gives the opportunity to determine separately the refractive index and the thickness of the layer. The same procedure is applicable whenever a very thin layer structure with two unknown parameters is under investigation.

Keywords: Protein adsorption, kinetics of; Protein adsorption, layer structure on fluid boundary; Ellipsometry on liquid interfaces; β-Casein

1. Introduction

Wide range of surfactants and stabilisers are used in food industry to control the formation and stability of emulsions. The proteins are inseparable part of many formulations [1]. The adsorption phenomena at a hydrophobic/hydrophilic liquid phase boundary are of primary interest for understanding the mechanism of protein action at the surface. Many studies have been devoted to the problem of protein adsorption and characterisation of the already formed layers, but a lack in studying the *kinetics* of adsorption still exists.

We have carried out a kinetic ellipsometric study of β-casein adsorption at two different interfaces (water/air and water/oil). The growth of the adsorption layer has been monitored, and the stages in the adsorption process are outlined. The

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0927-7765/00/$ - see front matter
PII: S0927-7765(99)00167-8

results for the final state of the layer are compared with the findings of other authors reported in the literature [2–5]. It is demonstrated that β-casein adsorbs in the same manner at the boundaries of oil and air phases with water; the already formed layer remains unchanged when the upper air phase is replaced by oil. The unique property of β-casein to form identical layers at two different interfaces is used to gain sufficient information (i.e. sufficient number of parameters), so as to determine the layer thickness and the refractive index.

It is well known that the ellipsometric angle ψ practically does not change from its value at a bare surface, when a very thin layer (100 times thinner than the light wavelength, λ) is formed on a substrate with real refractive index. In this case the first derivative of ψ with respect to the layer thickness d is zero, and the change of ψ from its value on a bare surface ($\delta\psi$) is proportional to the second-order term in the expansion over powers of (d/λ), namely, to $(d/\lambda)^2$ [6–8]. Thus, if no other information for the layer is available except that from a single ellipsometric experiment, the problem of finding the thickness and the refractive index cannot be solved, because only one parameter, the ellipsometric angle Δ, can be used. It is shown here that the information obtained by additional measurement in a different surrounding medium is sufficient to overcome this problem. The two unknown layer parameters — thickness and refractive index, can be determined from the two Δ values measured under different media. The method is valid even for thin multilayer structures with two unknown parameters. It is possible to solve numerically the inverse problem of ellipsometry, and to find these two parameters, using the two measured values of Δ.

We applied one and the same technique to study β-casein adsorption from water phase to the boundary with air and with oil, as the processes go in the same way. It is possible to show that the final state and the adsorption kinetics are virtually identical; thus, the layer thickness and refractive index are liable to determination continuously, in the entire experimental time interval.

The results from a 'single layer' model initially suggested by us indicate that it is not satisfactory for description of the real structure on the surface. Therefore, the model has been extended to a 'double layer' one, thus achieving a more accurate representation of the real system. We have found a very fast initial saturation of the surface with a thin dense layer of protein, and then the adsorption continues, forming a looser and thicker layer underneath.

2. Theoretical background

The ellipsometry gives the opportunity to study the processes of layer formation without direct contact with the samples, analysing only the polarisation state of the light reflected from the sample surface [6]. The change of polarisation state is described by two angles, Δ and ψ, which characterise the ability of the surface to reflect differently the light polarised perpendicularly and parallel to the incidence plane. The analysis of the experimental results is based on modelling the investigated system. The unknown parameters are usually found by a numerical minimisation procedure of fitting the 'designed' and the measured ellipsometric angles.

A problem with data interpretation appears when one measures very thin transparent layers (of thickness about hundred times smaller than the wavelength) on a nonabsorbing substrate [7]. In such a case only Δ is sensitive to the existing layer, while the angle ψ in a first approximation does not change from its value at the clean surface (without any layer). So, there are two unknown parameters (thickness, d_{1X}, and refractive index, n_X), and only one quantity coming from the experiment — Δ. Thus, the standard procedure for interpretation of the experimental data is not applicable, because of the insufficient number of informative parameters. As it was shown by Antippa et al. [7], for very thin transparent layers the following expression for the change of the ellipsometric angle Δ is valid up to the first order term in the expansion over powers of $d_{1X}/\lambda \ll 1$:

$$\delta\Delta = \Delta - \Delta_{\text{bare}} = \frac{4\pi}{\lambda}\frac{n_0 \sin(\varphi)\tan(\varphi)}{[n_2^2 - n_0^2][1-(n_0/n_2)^2\tan^2(\varphi)]}F_X = AF_X, \quad (1a)$$

$$F_X = d_{1X}\left(n_X^2 + \frac{n_0^2 n_2^2}{n_X^2} - n_0^2 - n_2^2\right). \quad (1b)$$

Here $\delta\Delta$ is the difference in the angle, for covered with a layer and bare surface, respectively, λ is the light wavelength, φ is the angle of incidence, and the refractive indexes n_0, n_X and n_2 refer to the substrate, the layer, and the immersion medium (upper phase). The thickness of the layer is d_{1X}, and A stands for the coefficient relating $\delta\Delta$ to F_X.

As it was mentioned above, for a transparent substrate (i.e. when n_0 is real) the change of ψ, $\delta\psi$, is zero up to the first order term in the expansion over powers of d_{1X}/λ. Thus, for thin layers we can only apply Eqs. (1a) and (1b) to find a connection between the film parameters. It should be pointed out that the multi-angular ellipsometry does not resolve the problem with the correlating layer parameters [7] (if one measures only $\delta\Delta$, then d_{1X} and n_X are not independent). Indeed, the coefficient A in Eq. (1a) depends on the angle of incidence, φ, the wavelength, λ, the substrate and the surrounding media refractive indexes, n_0 and n_2, but the layer parameters remain hidden in the term F_X (Eq. (1b)). The measured value $\delta\Delta$ is proportional to the term F_X, which contains the entire information about the layer. Thus, from a single ellipsometric measurement we can obtain the quantities n_X and d_{1X} built inseparably in the relation (Eq. (1b)).

However, the inverse problem for a thin layer is completely soluble if there are two values of $\delta\Delta$, measured with different surrounding phases. As the refractive index of the upper phase, n_2, is included in the expression (1b), the corresponding two formulae for F_X can be combined and solved for n_X and d_{1X}. The easiest way to gain two independent F_X values is to change the surrounding medium from air to oil, thus the refractive index n_2 changes from $n_2 = 1.00$ (air) to the value of the oil. We used pure xylene (isomeric mixture), with $n_2 = 1.50$.

The real situation is a bit more complicated, because instead of one new phase we have two phases — thick oil layer, and air on top of the sample. The light beam which reflects from the investigated surface passes twice through the air-oil boundary. The additional analysis (not given here) shows that the ellipsometric angle Δ is not influenced when the light beam passes through a flat interface between transparent media. Only a correction for the incidence angle φ is needed, because the beam refracts at the air/oil boundary and comes to the layer at a smaller angle. Accordingly, we use the Snell's law.

The substrate is water solution, and air and xylene are the surrounding media in our protein adsorption experiments. The adsorbed layer refractive index is determined by using the ratio of both terms F_X for $\delta\Delta$ measured in air and under oil:

$$\frac{(\delta\Delta_o)A_a}{(\delta\Delta_a)A_o} = \frac{n_X^2 + (n_o^2 n_w^2/n_X^2) - n_o^2 - n_w^2}{n_X^2 + (n_a^2 n_w^2/n_X^2) - n_a^2 - n_w^2}, \quad (2)$$

where the indices a, o, and w designate air, oil, and water, respectively. Eq. (2) determines the refractive index n_X, and then from Eqs. (1a) and (1b) d_{1X} can be calculated.

The procedure of combining the ellipsometrical data under different immersion media can be successfully applied even for very thin layer *structures* with two unknown parameters. Then, a numerical minimisation procedure should be used for fitting the modelled and the measured Δ values (there will be no analytical solution connecting n_X with $\delta\Delta_o$ and $\delta\Delta_a$, such as that discussed above — Eq. (2)). An example of this kind of treatment is given in the present paper, where formation of a second layer beneath the first adsorbed one takes place. The refractive index and the thickness of the second layer in its final state are found from the measured ellipsometric angle Δ under air and oil, using the already known thickness and refractive index of the first layer. A minimisation procedure for finding the parameters of the second layer is applied, according to the double layer model (see below).

Finally, an important point should be mentioned: the parameters of the thin layer themselves (n_X, d_{1X}) should not change when the surrounding medium is replaced by another phase. Otherwise, the procedure proposed above will be meaningless.

3. Experimental

3.1. Apparatus

A null type ellipsometer [6] — 'polariser compensator sample analyser', with a He–Ne Laser source ($\lambda = 632.8$ nm), and a photo-multiplier as a detector of the reflected light intensity were used. The measurements were carried out at two different incident angles with respect to the surrounding media — 70 and 50° for oil and air, respectively. The procedure of diminishing the output light signal in 'two zone' method [6] allows us to measure Δ with accuracy of 0.06, and ψ with 0.006° (for optimal conditions on a stable solid surface), and to perform sampling every other minute. Thus, the initial (relatively fast) kinetics of casein adsorption was followed in 2-min intervals. At longer times the kinetics becomes slower, then the measuring intervals were increased, according to the changes in the ellipsometric angle Δ.

3.2. Materials

Lyophilised, essentially salt-free β-casein from bovine milk, purchased from Sigma Chemical Co. (Catalogue # C-6905), was used as received. Other substances, such as hydrochloric acid to adjust the pH, and sodium azide to prevent bacterial contamination, were added to the water solutions of protein. All samples were prepared with deionised water obtained from a Milli-Q system (Millipore). Purified xylene was used as a hydrophobic oil phase. It had been preliminarily filtered through a column packed with chromatographic adsorbent (Florisil), in order to remove any surface-active impurities.

3.3. Procedure of sample preparation

The adsorption studies were carried out with aqueous solution of 0.01 wt.% β-casein containing 0.2 g/l sodium azide (NaN_3), at pH 5.0 adjusted with hydrochloric acid. Purified xylene was used as a model hydrophobic phase. Although it is inapplicable in the food industry, we wanted to prevent contamination with fatty acids, lipids and other surface active components usually present in natural oils, that is why xylene was preferred.

Three kinds of experiments were carried out.

1. *Adsorption of β-casein at air–water interface*. The solution with the desired concentration was initially prepared and poured into a glass vessel. Then, the sample was aligned to the light beam at the ellipsometer sample stage. The measurement was started immediately after cleaning the surface (the latter was accomplished by sucking out with a small pipette).
2. *Adsorption of β-casein at oil–water interface after protein injection in the aqueous phase*. Initially, water with the desired pH (without protein) was covered by a thick oil layer (~ 2 cm). The obtained bare o–w interface was aligned to reflect the light beam. Then, a concentrated β-casein solution was injected into the water phase. The protein quantity was chosen in such a way as to ensure the desired bulk concentration in the whole volume of the aqueous phase. The measurements started immediately after the injection.
3. *Measurements of initially formed β-casein layer, after changing the air–water interface to oil–water interface*. A protein layer was initially formed at the air–water interface. Then, oil was carefully poured on top of the protein layer. The changes in the layer were afterwards monitored.

The kinetic curves, $\delta\Delta$ versus square root of time, are given in Fig. 1. The difference in the ellipsometric angle Δ from its value at the bare interface ($\delta\Delta$) is presented. The time scale $\sqrt{t}$ is chosen for a better illustration of the kinetics. The values of $\delta\psi$ are not shown, because the experimental error is far greater than the effect from the layer formation. The measurements have been carried out at room temperature (18 ± 1°C), and were reproducible within the data scatter shown on Fig. 1.

The scattering of the raw experimental data ($\delta\Delta$ on Fig. 1) is relatively high, which is to be expected when ellipsometry is applied to *fluid* interfaces. The reasons are connected with the liquid surface vibrations, and with the small reflected intensity. These problems become even more pronounced for liquid/liquid/fluid systems (in our

case, water/oil/air), when the lower boundary is being investigated. The intensity of the light reflected from the water/oil interface is very low, and moreover, one has to separate this beam from the much more intensive beam primarily reflected from the oil/air surface. As mentioned in Section 3.1, the accuracy of the ellipsometric method itself (and of the apparatus) is about 0.06° in Δ (which would have been realised on a stable solid surface). In reality, the experimental data scattering

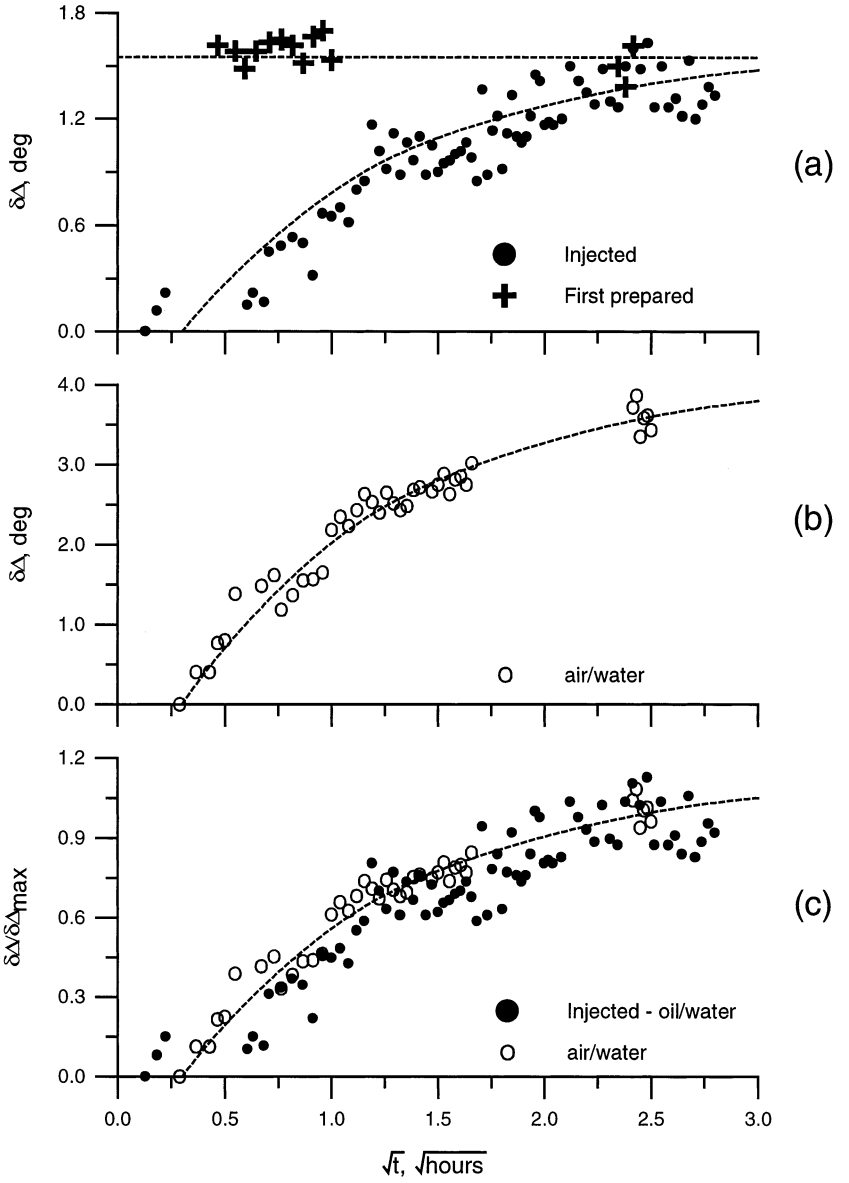

Fig. 1. Raw ellipsometric data. (a) Layer under oil, prepared by injection (method (II) in Section 3.3, the full circles), or by pouring oil over a layer initially formed on air/water surface (method (III), the pluses); (b) results for air/water interface (method (I) in Section 3.3); and (c) the data for $\delta\Delta$ from the case (a) (the full circles) and the case (b) are scaled by the respective average final value of $\delta\Delta$ (denoted by $\delta\Delta_{max}$), and are plotted together.

is 3–4 times greater than the value 0.06° (Fig. 1).

4. Discussion

The analysis of the protein adsorption behaviour, as seen from the raw ellipsometric data, leads to the following three conclusions.

1. The layer initially formed on air/water boundary and after that poured over with oil (Fig. 1a — bold plus symbols) does not exhibit any change with time. We accept that the adsorbed layer is stable, and is not disturbed by exchange of the upper phase. Eventual reconfiguration should have finished completely within those several minutes needed for aligning the sample stage after the oil addition and preparing for the measurement. The period during which the sample was not ellipsometrically monitored (2–4 min) is much shorter than the characteristic times of adsorption ($\sim$20 min), so we are convinced that no layer transformation takes place.
2. The layer formed on the bare oil/water surface after injection (Fig. 1a — full circles), reaches the same Δ as the layer initially formed in air and then poured over with oil ($+$ signs in Fig. 1a). The equal final state, irrespective of where the layer was formed initially, testifies for the insignificant role of the oil: it does not influence the final state of the protein. Hence, there is no substantial hydrophobisation of the adsorbed β-casein molecules, or at least the thickness and the refractive index of the layer are not affected.
3. The kinetics of layer formation on air/water interface (Fig. 1b — open circles), compared to the kinetics on initially bare oil/water interface (Fig. 1a — full circles) shows similar time behaviour for both samples, but different magnitudes of $\delta\Delta$ (because the oil and the air have different refractive indexes). The two curves in Fig. 1(c), obtained by scaling of those from Fig. 1(a, b) by normalising with the maximum $\delta\Delta$, have identical shape.

In other words, the final state of the layer formed initially in air (Fig. 1b) does not differ from that of layer formed by injection under oil (Fig. 1a — circles), because when immersed into oil the former gives the same Δ as the latter (Fig. 1a — $+$). The equal final state, and the similar kinetics beneath oil and air, lead to the conclusion that the protein adsorption and conformation do not depend on the surrounding hydrophobic phase, be it oil or air. This inference should not seem surprising, because β-casein is known to be a disordered molecule which possesses no tertiary structure and S–S linkages, with little ordered secondary structure only [2].

There are now two separate parameters available to determine the layer state — the two angles Δ measured under oil and under air.

To work out a single-layer model for interpretation of our experimental results, we applied a linear interpolation of the data from the two curves $\delta\Delta(t)$, in the following manner: First, we sort all points in order of increasing time. Next, for a given point from one $\delta\Delta(t)$ curve we find a value of $\delta\Delta$ which corresponds to the same instant of time on the other $\delta\Delta(t)$ curve, by interpolation between the two nearest measured points on the latter curve. Thus, values referring to one and the same time moment (under air and oil) were obtained. Then, the procedure for determination of n_X and d_{1X} described above was implemented, at each couple of corresponding points. Thus, the thickness and the refractive index were found as functions of time.

Each (n_X, d_{1X}) couple now yields the respective protein volume density (concentration) in the layer, c, and the surface concentration (the adsorption, Γ). c is calculated from the linear approximation for the refractive index of protein solutions:

$$n_X = n_w + \left(\frac{dn}{dc}\right)c, \tag{3a}$$

where $dn/dc = 0.18$ cm^3/g (literature data, cf. Refs [3,9]). The volume density (layer concentration), c, and the layer thickness, d_{1X}, determine the adsorption, Γ:

$$\Gamma = cd_{1X}. \tag{3b}$$

The linear type of the relation connecting the refractive index with the volume concentration,

Eq. (3a), imposes a restriction on the range of its applicability. Indeed, Eq. (3a) has been established by experimental measurements of n in bulk aqueous solutions (not in interfacial layer). So, to apply Eq. (3a) for the layer, one has to extrapolate bulk data obtained at relatively low concentration of protein to the very high concentrations inside the adsorbed layer [5].

4.1. Results for the single layer model

Supposing a homogeneous layer formation during β-casein adsorption, and applying the treatment described above, we calculate the curves $n(t)$, $d(t)$, $c(t)$, and $\Gamma(t)$. The results are presented in Fig. 2.

The refractive index increases rapidly at the very beginning, and reaches the value of 1.45 for 21 min. Then, it tends to decrease back to ~ 1.40, during the entire measuring interval. The thickness shows different behaviour. It increases monotonously over the whole time interval, approaching the value of 6 nm. At the critical point of the refractive index curve (the maximum), d has a value of 1.8 nm. As the volume concentration, c, is proportional to the refractive index, its curve has the same behaviour as that of n. The adsorption Γ steadily increases, up to 2.3–2.5 mg/m^2 at the end. This value is of the same order as the values reported in the literature. Graham and Philips [3] obtained a saturated coverage of 2.6 mg/m^2 (measured by surface radioactivity), and 4 mg/m^2 by ellipsometry (with β-casein after more than 16 h). More recent study by radiotracer technique [4] gave 2.2 mg/m^2 for β-casein layers adsorbed from solutions with concentration in the range 10^{-5}–10^{-4} wt.%. The protein concentration in our experiments is considerably higher (10^{-2} wt.%), but this influences mostly the rate of adsorption and not its final state. The latter conclusion follows from the fact that our Γ (2.3–2.5 mg/m^2) practically coincides with that from Ref. [4]. According to Ref. [3], Γ does not depend on the bulk concentration in the range 10^{-4}–10^{-2} wt.%.

The fast initial increase of the refractive index (and volume concentration), and the relatively stable further values, indicate rapid adsorption and ordering of the protein molecules at the bare surface, and formation of a tight layer up to the 21st min. Afterwards, the layer starts to grow thicker and a bit more dilute (Fig. 2b, c). Additional protein attaches to the surface during the thickness increase, which is proved by the rising values of Γ (Fig. 2d).

The peculiar behaviour of the refractive index, which diminishes after the 21st min, can be explained by the hypothesis that, first, a rigid thin layer forms, up to complete surface coverage, and then the protein from the bulk keeps adsorbing underneath slowly, building a second layer. It is reasonable to assume that the first process is irreversible [3], and the first monolayer keeps its density constant. The maximum in the volume concentration curve can be interpreted as the final stage in the fist layer formation. Next, this process of fast irreversible formation of the first monolayer is followed by a continuing slower adsorption of molecules coming from the bulk. The structure built up in this way has two different sub-layers, with distinct optical properties. The above considerations lead us to the conclusion that the 'single layer' model can be taken only as a first approximation, and should be extended to a more accurate model of double layer structure.

The 'single layer' model is adequate (in the sense of representing the real structure) up to the critical point on the refractive index kinetic curve (the maximum). A dense monolayer initially forms alone at the surface, and only then the further underneath adsorption commences. Hence, the first layer in the more elaborate model will have well defined parameters liable to direct determination: its refractive index and thickness correspond to the 21st min (the *maximum*) on the kinetic curves in the single layer model. The kinetics within the rest of the experimental time interval is treated as growth of a second looser layer.

These ideas are supported by the available information in the literature about the structure of β-casein molecule and its conformation on fluid interfaces. β-casein is a single-chained protein (molecular weight 24 000), composed of 209 amino-acid residues with known sequence [2,10]. The molecule has little ordered secondary struc-

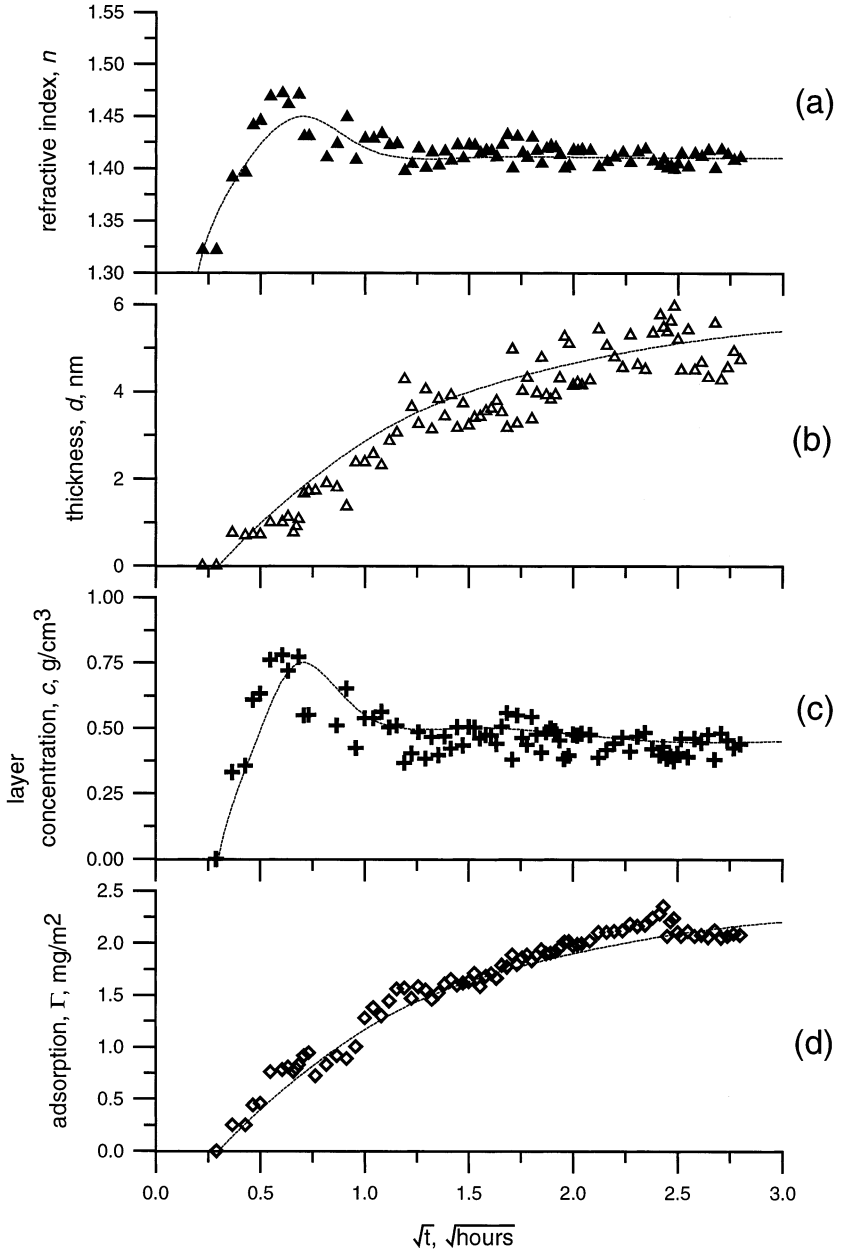

Fig. 2. Calculated results for one-layer model. The curves for the refractive index (a), and the volume concentration (c) exhibit maxima. This may be considered as a result from formation of a first very dense monolayer of protein. The subsequent diminishing after the maxima in both curves indicates formation of a second looser layer. The thickness and the adsorption (curves b, d) increase monotonously.

ture, and does not contain disulphide bridges and tertiary structure [2]. The β-casein molecule has a distinctly amphiphilic nature, because the first 50 amino-acids (starting from the N-terminus) are hydrophilic, whereas the remaining 159 residues are predominantly non-polar and rather hydro-

phobic [2,10]. This determines the ability of β-casein to aggregate in aqueous solutions, forming the so called 'sub-micelles' with diameter 15–20 nm [11]. The size of the sub-micelles (Ref. [11]) is in good correlation with the thickness of thin aqueous films between xylene phases, about 20 nm, measured in a capillary cell [10]. Such films are actually bilayers.

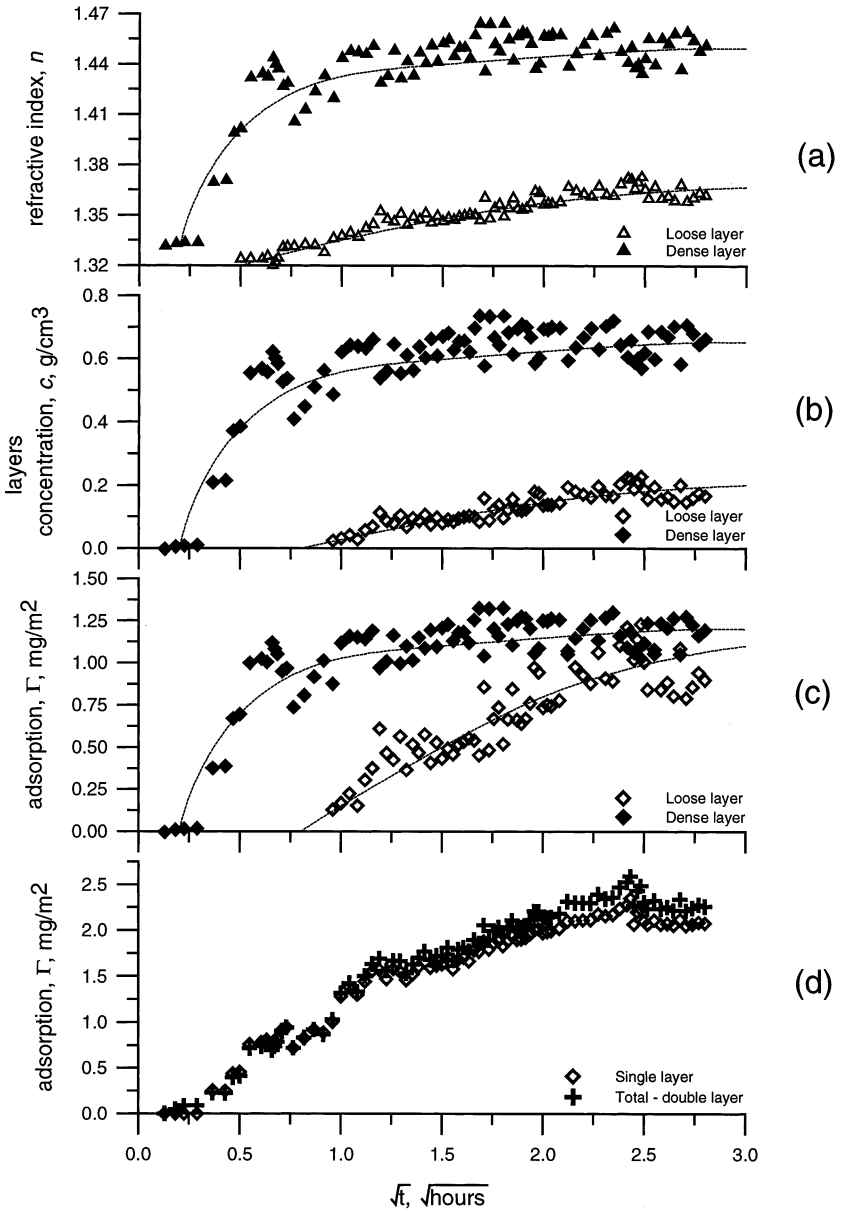

Fig. 3. Results from the double layer model. (a–c) Parameters of the two substructure layers. The first one forms rapidly, and retains constant values of n (refractive index), c (volume concentration), and Γ (adsorption). The second sublayer develops slowly underneath. Case (d) comparison between the results for Γ calculated from the single layer model (case d in Fig. 2) and the double layer model (the sum of the two curves in Fig. 3c).

Table 1
Final state in the double layer model — Fig. 4[a]

Substituted in the minimisation procedure				Calculated results	
$\delta\Delta$ (oil)	$\delta\Delta$ (air)	n (1st layer)	d (1st layer)	n (2nd layer)	d (2nd layer)
1.44°	3.60°	1.45	1.8 nm	1.366	5.4 nm

[a] Final state parameters for double-layer model, calculated from (i) the final $\delta\Delta$ measured under oil and air; and (ii) the known thickness and refractive index of the first dense layer.

Graham and Phillips [12] discussed in detail the conformation of β-casein adsorbed on fluid boundaries. Those authors considered it likely that at low surface pressure (and coverage) the molecules lie flattened on the interface in an 'all-train' configuration (i.e. most residues are located in the plane of the surface). With progressively increasing Γ more loops and tails form. Such a view may explain why in our experiments initially the layer is very thin (~ 1.8 nm) — it probably contains only molecules in 'side-on' position. Afterwards, as new protein molecules come to adsorb, more segments protrude in loops, and the hydrophilic portions of the chains dangle into the water phase as tails. Thus, the thickness increases gradually.

Neutron reflectivity study of β-casein adsorption [2,5] gives a structure of two layers — dense hydrophobic 'inner' surface layer of thickness 1.85 ± 0.15 nm, which is closest to the liquid boundary, and more diffuse 'outer' layer (from the side of the aqueous solution), of thickness 6.85 ± 0.74 nm. The double layer model reported here (Section 4.2) leads to approximately the same picture for the final state of the layer; the calculated total surface concentration (Γ) is the same as that in the single-layer model.

4.2. Results for the double-layer model

4.2.1. Final state

The final state of the double-layer structure was calculated using the already known parameters for the inner dense layer, as they were found at the 21st min with the single-layer model. A minimisation procedure for the thickness and the refractive index of the second (looser) layer was applied to the final values of $\delta\Delta$, measured under oil and air. We vary n and d of the second layer until the theoretically predicted two values of $\delta\Delta$ (for oil and air) coincide with those measured experimentally. Table 1 summarises the results. The thickness and the refractive index of the second layer, calculated in this way, are in agreement with those given in Ref. [5].

4.2.2. Kinetics of second layer formation, with known parameters for the first layer

Double-layer model treatment was attempted to the experimental data for $\delta\Delta$ in the time interval from the 21st min to the end of the measurements. We searched for the thickness and the refractive index of the second layer, substituting with the parameters of the first layer (determined at the 21st min). This procedure, however, led to rather scattered values which could not be interpreted. We suppose this is as a result of the undefined boundary between the substrate and the diffuse layer at the time of its formation. The deviations in the thickness and in the refractive index correlate, so that nearly smooth curve is obtained for the kinetics of the surface coverage (Γ). The values of Γ for the second layer, obtained in such a way, are very close to those found in Section 4.2.3 below.

4.2.3. Kinetics of formation of two layers with constant thicknesses

To overcome the above mentioned difficulty, we decided to consider two layers at fixed thicknesses: 1.8 nm for the first layer and 5.4 nm for the second one (see Fig. 4 and Table 1). Then, a numerical procedure was applied to solve for the two unknown refractive indexes of the two sub-

structure layers. We chose a model with initially known thicknesses because it will give us the possibility to check whether the refractive index of the first layer changes with time, and in this way to validate the suitability of the double layer model. By assuming fixed thicknesses of the first and second layer we actually consider a fictitious 'empty' structure. The determination of the two refractive indexes (by minimisation procedure) gives us how this structure is filled up with protein as time passes. Thus, we shall be able to find how much protein goes to the first thin layer, and how much to the second thicker one, at each moment of time.

The calculated refractive indexes, n, surface concentrations, Γ, and volume densities, c, of the two layers are given in Fig. 3. The dense inner monolayer forms first and then keeps its parameters constant with time (note the plateaux in the curves a, b and c). The second loose layer starts to develop almost immediately after the first one. The first 'inner' layer is three to four times denser than the submerged 'outer' one, and has approximately constant surface concentration ($\Gamma = 1.2$ mg/m^2), over the whole experimental time interval after the initial formation. The total protein surface concentration ($\Gamma(t)$) curves for the single and double layer models are practically identical (see Fig. 3d), which provides evidence for the self-consistence of our treatment.

Our results for the total protein adsorption (surface concentration, Γ, in the final state, Fig. 3d), are quite close to the values given in Refs. [3,4], measured by surface radioactivity and radiotracer method (2.6 and 2.2 mg/m^2, respectively). A limitation in the applicability of our model could be brought about by the restricted validity of the linear approximation for the dependence $n(c)$ — Eq. (3a), as it holds (and was experimentally proven with bulk solutions) at low protein concentrations, but is extrapolated to rather high protein concentrations such as those in the dense layer [3]. Still, our value for the volume concentration in the second diffuse layer ($c = 0.2$ g/cm^3) does not differ significantly from that reported in Ref. [5] (0.26 g/cm^3).

5. Conclusions

Ellipsometry was applied to monitor the β-casein adsorption in situ, at two different interfaces — air–water and xylene–water. It is shown that the layer formation is not significantly influenced by the type of the hydrophobic phase. In both cases the adsorption starts with saturation of the surface with a thin dense layer, and continues further, forming a thicker and looser second layer. The thicknesses of the two layers are close to those obtained by neu-

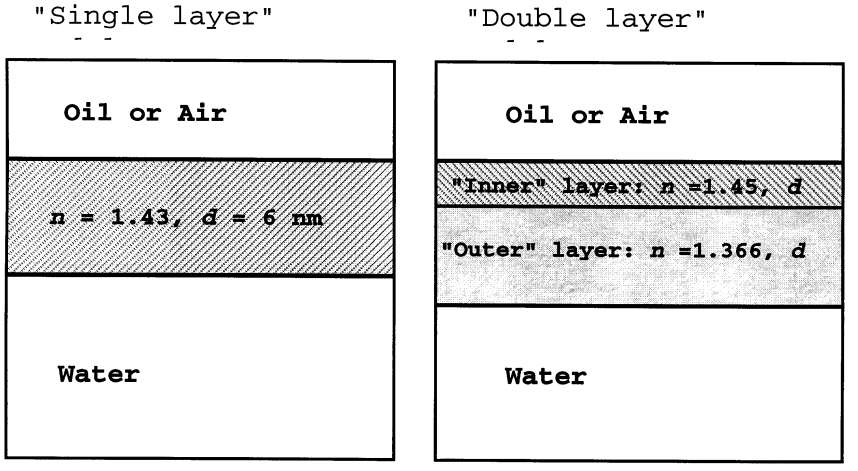

Fig. 4. Sketches of the interfacial layer in the frames of the two models used to interpret the ellipsometric results.

tron reflectivity studies (reported in the literature).

The additional information needed to solve the inverse ellipsometric problem in the case of a very thin layer can be gained by measurements under two different media (oil and air). This gives the opportunity to determine two unknown layer parameters (e.g. the thickness and the refractive index, if a single-layer model is adopted). We calculate the values of the adsorption (Γ), and the volume concentration of β-casein layers, using linear approximation for the dependence of the refractive index on the concentration. The results for Γ are in good agreement with those measured by other authors, by surface radioactivity methods.

Acknowledgements

The authors wish to thank Kraft Foods, Inc., USA, for the financial support of this work.

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