Thin Solid Films, *238* (1994) 88–94

Reversed micellar approach as a new tool for the formation and structural studies of protein Langmuir–Blodgett films

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(Received May 13, 1993; accepted July 21, 1993)

Abstract

Reversed micelles of sodium diisooctylsulfosuccinate with cytochrome c were used as spreading solutions for Langmuir–Blodgett film formation. A condensed monolayer with closely packed surfactant molecules in the air and closely packed protein molecules under the water surface was formed after several repetitions of the compression–expansion cycle. The resulting film was transferred onto the solid substrate. The films were studied by circular dichroism and by small-angle X-ray scattering. It was shown that the metalloprotein in the film is not denatured and the resulting thin film is significantly ordered, more so than the corresponding monocomponent protein Langmuir–Blodgett films.

1. Introduction

The idea of bioelectronics, presently widely discussed, requires the utilization of very thin regular protein layers organized in three-dimensionally ordered structures with passive molecular supports (conductive layers, insulators). One of the most promising methods is to apply the Langmuir–Blodgett (LB) technique [1]. At present it seems to be the best procedure which allows functional structures with molecular resolution to be constructed. Owing to its relatively mild process conditions, the technique was successfully applied to the fabrication of films of several proteins [2, 3].

It was found that the LB method may be applied in a classic way when working with membrane proteins, such as photosynthetic reaction centres and bacteriorhodopsin [4, 5]. It is also possible to utilize this method for some of the water-soluble proteins which are stable at the air/water interface. Immunoglobulin G is one of the examples of this type of protein [6]. However, water-soluble proteins, the structures of which are maintained only by polar and hydrophobic interactions, tend to denature at the air/water interface. The forces that sustain the structure of such proteins are of the same order of magnitude as the surface tension, thus, after such a protein is placed on the water surface, the action of the surface tension unfolds the protein by disrupting the existing intramolecular interactions. (With the exception of immunoglobulins, which are stabilized by covalent S–S bonds within the molecule. These bonds are much stronger than the surface tension which preserves the molecular structure from denaturation.)

Since many of the water-soluble proteins have significant functional interest for many fields of natural science (for example, cytochromes which are promising objects for bioelectronics [7]), a search for the ways of applying the LB method to these proteins has been initiated. The activities mainly took two directions, namely the utilization of charge [8] and specific [9] interactions. In both cases a monolayer of lipid was formed at the air/water interface. In the former case the heads of lipid molecules were charged oppositely to the protein surface, and in the latter case the lipid head groups had a specific affinity to some regions of the protein molecules. The protein molecules, once injected under the monolayer, were regularly adsorbed at the lipid head groups.

As a result, the formation of 2D crystalline protein domains was observed. However, the techniques used up to now also have certain disadvantages. One of the most important facts is that during adsorption at the lipid/water interface a non-homogeneous distribution of protein in the film was created, a fact that has been observed by fluorescence microscopy [9]. Besides regular protein crystal domains, some irregularities such as empty regions without protein molecules, have been observed. The second disadvantage is that not all of the protein molecules are bound to the lipid head groups

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0040-6090/94/$7.00

due to simple solubility of the protein in the aqueous phase and, hence, redistribution of a major part of protein molecules into the bulk solution.

The present approach was suggested to avoid the above disadvantages. In earlier studies a glucose oxidase molecule was surrounded by about 200 lipid molecules in such a way that the protein was in the middle of the particle (in contact with the lipid head groups) and hydrocarbon chains were exposed outside [10]. Such particles were dissolved in organic solvents and the LB technique in the classic version, *i.e.* spreading of organic solution, compression and transfer, was applied. The experiments have shown that it was thus possible to deposit glucose oxidase molecules and that the protein did not lose the enzymatic activity in the LB film. However, the use of long chain lipids resulted in a non complete dense packing of the proteins due to the fact that the area covered by lipids was larger than the area covered by protein molecules.

Here we will apply the reversed micellar approach [11] to the construction of cytochrome c containing LB films. Major attention will be paid to the preservation of the secondary structure of the protein in the LB film and to the molecular organization of the film.

2. Experimental details

The protein, cytochrome c from horse heart, was obtained from Sigma. Prior to use, the commercial preparation was dissolved in phosphate buffer, 25 mM, pH 7.5, dialyzed against the same buffer, and concentrated. The obtained solution was used further at a concentration of 6.8 mg ml $^{-1}$.

Sodium diisooctylsulfosuccinate (Aerosol OT, AOT) and n-octane were obtained from Fluka and used without further purification.

Reversed micelles of AOT in octane were prepared according to the standard procedure described elsewhere [7]. Cytochrome was solubilized at the hydration degree of 8.6 (for explanation see Section 3).

Monolayer studies and the transfer of the films onto solid substrates were performed in a MDT LB trough (Russia). The trough size was 240 x 100 mm^2 and the volume was 150 ml. The system allows for bilateral compression, with the surface pressure, surface potential, and deposition point appliances positioned in the centre of the trough. The surface pressure sensitivity was 0.05 mN m^{-1}. Surface potential measurements were made with a vibrating electrode, and the sensitivity was 10 mV.

Water purified by the MiliQ system was used as a subphase (resistivity greater than 18 MΩ cm).

The monolayers were transferred onto solid substrates using the horizontal lift technique (Langmuir–Shaefer method) [2]. Chemically polished silicon wafers were used as substrates for the deposition of the LB films for X-ray study and quartz slides (Suprasil, Hellma) were used as supports for circular dichroism (CD) studies.

Structural investigations were carried out on a small-angle X-ray diffractometer with a linear position-sensitive detector [12]. The measurements were taken with an angular resolution of 0.02° (the resolution in the spacing determination was 0.05 nm).

CD measurements were made on a Jasco J-710 spectropolarimeter at a protein concentration of 0.5 mg ml^{-1} in the 190–260 nm range at a resolution of 0.1 mdeg cm^{-1} and a time constant 8 s. The instrument was calibrated for inactive sample artefacts with a known standard.

3. Results

As a first step, cytochrome c was solubilized in the reversed micelles of AOT in octane. Since the extent of polar interactions is determined by the surface area of the contact, apart from other factors, it was feasible to select a point of spatial compatibility between the protein molecule and the micelles.

The inner surface of the spherical cavity of reversed micelles is negatively charged owing to the presence of the head groups of the surfactant. When a globular protein is solubilized in the reversed micelles, it is surrounded by the surfactant molecules that are thus interacting with the surface charges of the protein globule. The smaller the distance between the polar heads of surfactant molecules and the protein surface, the stronger the resulting interaction.

The dimensions of the inner water cavity of AOT reversed micelles are determined solely by the water content in the system, other conditions being unchanged. The water content is characterized by a parameter called the "hydration degree", or w_0, which is a molar ratio of water to surfactant concentrations in the system [7]. Thus, choosing a micellar matrix into which the protein "fits" tightly, should provide proper contact of the protein and surfactant molecules along the whole inner surface of the reversed micelle.

Cytochrome c, which is a globular protein with a molecular weight of about 12.5 kDa, is sufficiently well approximated by a sphere with a diameter of 3.4 nm [13]. The hydration degree of 8.6 corresponds to almost the same diameter of the inner cavity of micelles. This was exactly the value which was chosen for the solubilization of the enzyme prior to spreading at the water subphase.

Deposition of a small droplet of the reversed micellar solution onto the water surface resulted in a marked

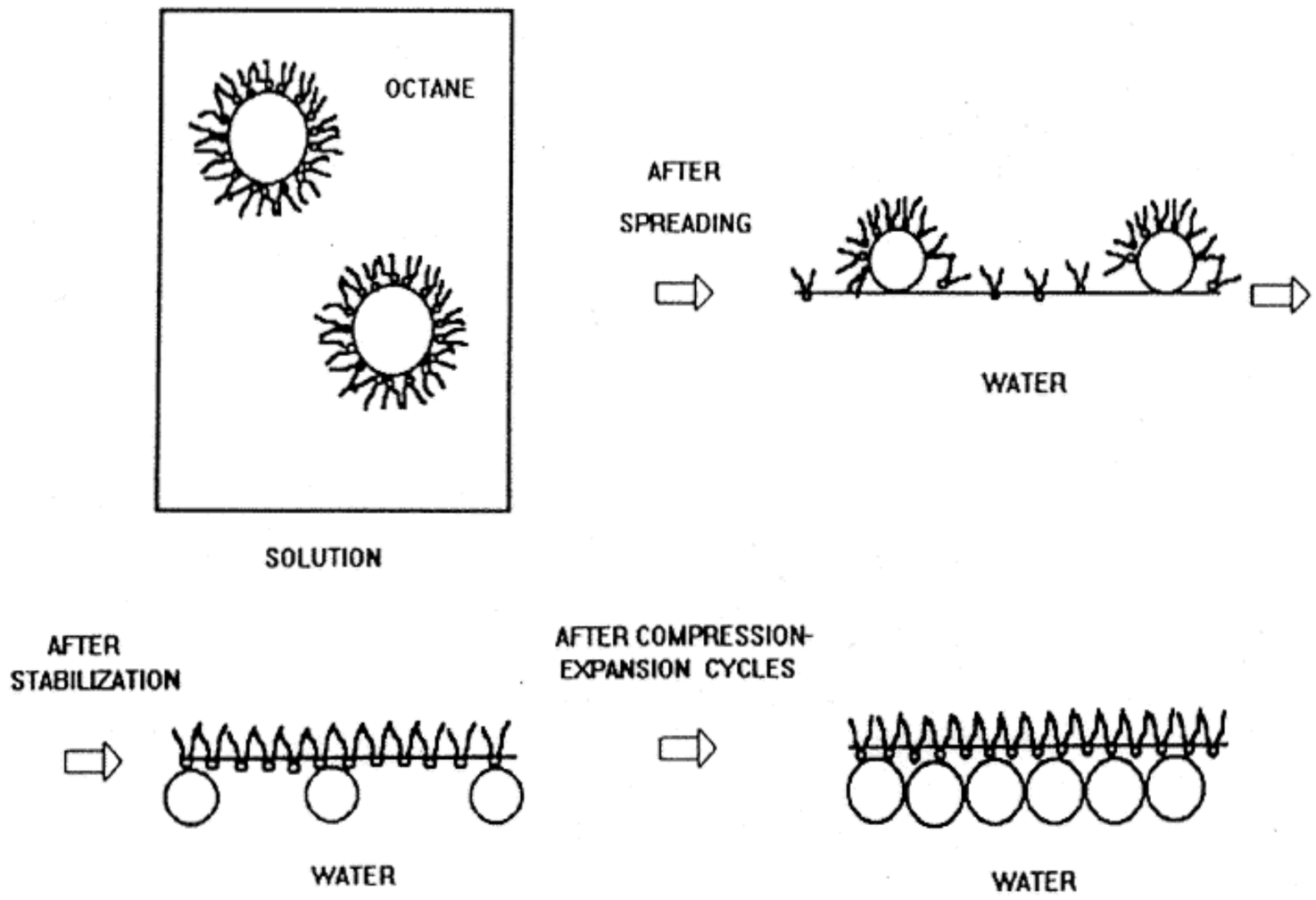

Fig. 1. Model of the formation of LB films using reversed micellar solutions. Initially, cytochrome c molecules are solubilized in the solution of AOT reversed micelles in octane. Spreading of the solution reorganizes the micelles in such a way that AOT molecules form a monolayer at the air/water interface and cytochrome molecules are attached to their head-groups. Application of compression–expansion cycle several times results in the removal of excess molecules of AOT from the water surface and in the formation of closely packed monolayers both of AOT at the interface and of proteins in the water in the vicinity of the surface.

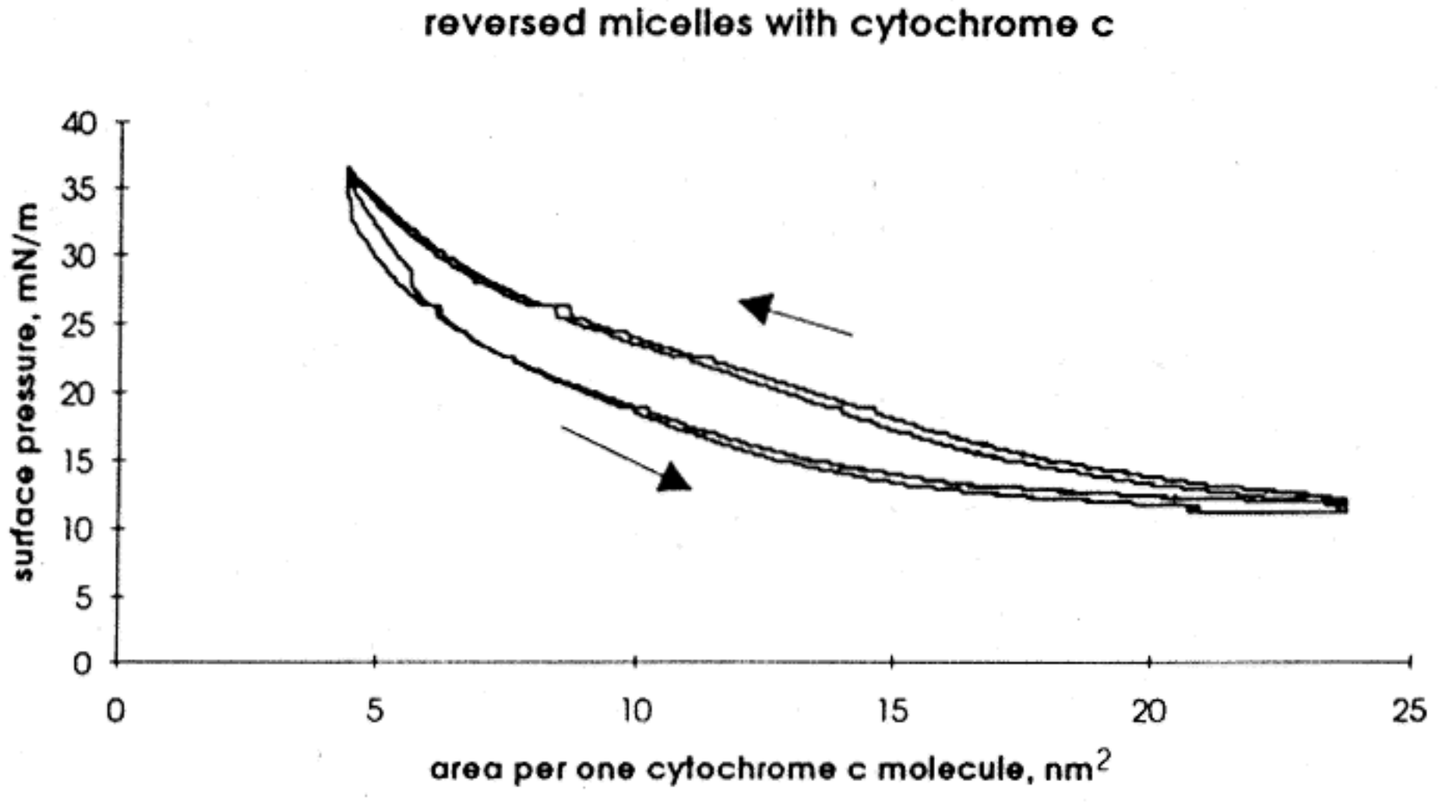

Fig. 2. π A isotherm of the monolayer of reversed micelles with cytochrome c after four compression–expansion cycles. Arrows indicate the direction of the barrier motion (compression or expansion).

increase of the surface pressure (deposition of a 10 μl droplet resulted in an increase in the surface pressure of about 20 mN m^{-1}). This should be due to the reorganization of the micelles at the air/water interface in such a way that the AOT molecules of the monolayer are turned with the hydrophobic tails from the water surface, and the protein molecules are bound to the surfactant head groups under the water (Fig. 1).

The $\pi-A$ isotherm of a spread reversed micellar monolayer is presented in Fig. 2. During initial compression–expansion cycles the curve was not reproducible, probably as a result of the stabilization processes taking place in the film. The reproducibility of the $\pi-A$ isotherm was achieved after four compression–expansion cycles.

The $\pi-A$ isotherm of "empty" reversed micelles of AOT (without cytochrome) is similar to that of the reversed micelles with cytochrome c. Thus, the main reason for the increase of the surface pressure is mainly the presence of the surfactant at the air/water interface.

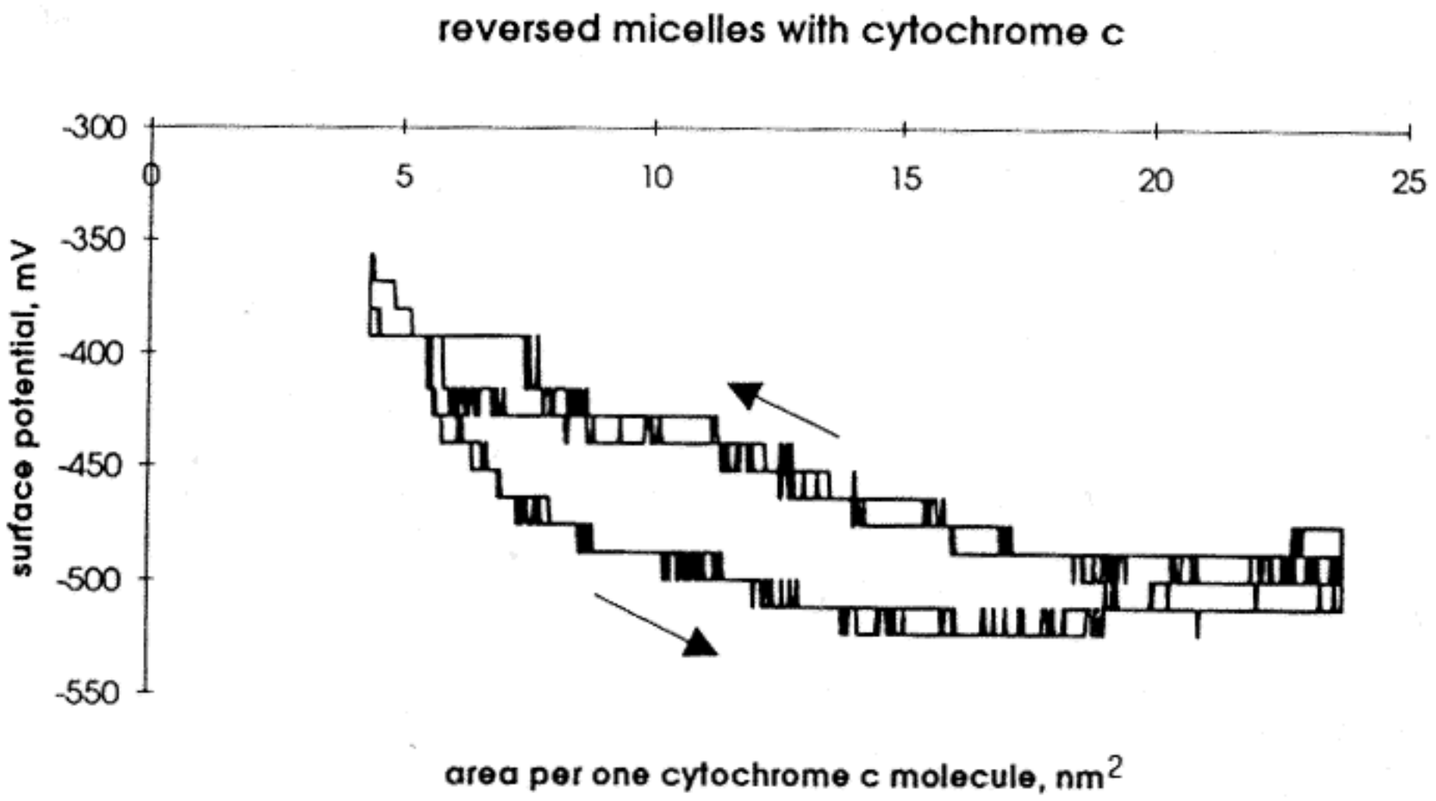

Fig. 3. The dependence of the surface potential of the AOT–cytochrome c monolayer with respect to the area per one cytochrome c molecule (two cycles of compression–expansion). Upper curves, compression; lower curves, expansion.

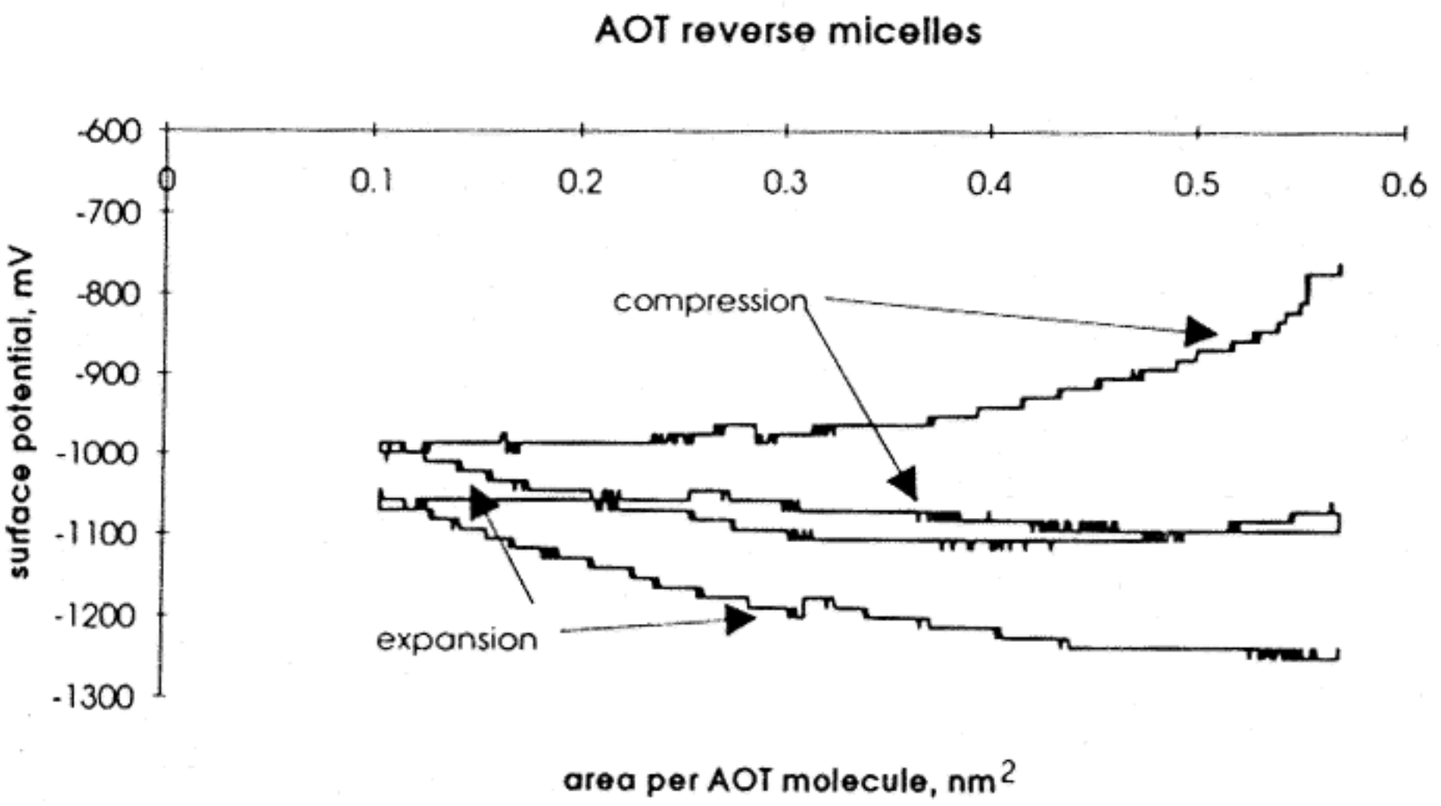

Fig. 4. The dependence of the surface potential of the AOT monolayer (reversed micelles without cytochrome c) with respect to the area per AOT molecule (two cycles of compression–expansion). The small value of the area per molecule indicates that penetration of AOT molecules into the water volume takes place immediately after spreading of the monolayer.

The presence of the protein in the system can be registered by surface potential measurements. The dependencies of the surface potential of the reversed micelles with and without cytochrome c are presented in Figs. 3 and 4, respectively.

It is evident from Fig. 4 that for reversed micelles which do not contain protein molecules, the surface potential decreases continuously in the expanded region after each compression–expansion cycle. This could be due to the fact that the surface concentration of the surfactant decreases after each cycle. The AOT monolayer is not stable at the air/water interface and each compression–expansion cycle might result in a decrease of the effective amount of surfactant molecules at such an interface. (Most probably the rest goes over into bulk solution in the form of normal micelles at least in the proximity of the interface where the effective concentration should be higher than the critical concentration of normal micelle formation. However, it is possible that separate surfactant molecules dissolve in the solution, or that micro collapses are formed in some regions of the monolayer.)

Protein-containing reversed micelles do not exhibit similar behaviour, *i.e.* a continuous decrease of the surface potential was not observed. The curves are reproduced during repeated compression–expansion cycles (Fig. 3), which proves the surfactant–protein complex is more stable at the air/water interface and, since the surface potential values per single molecule area are reproduced, implies that this complex constitutes the relatively stable monolayer unit.

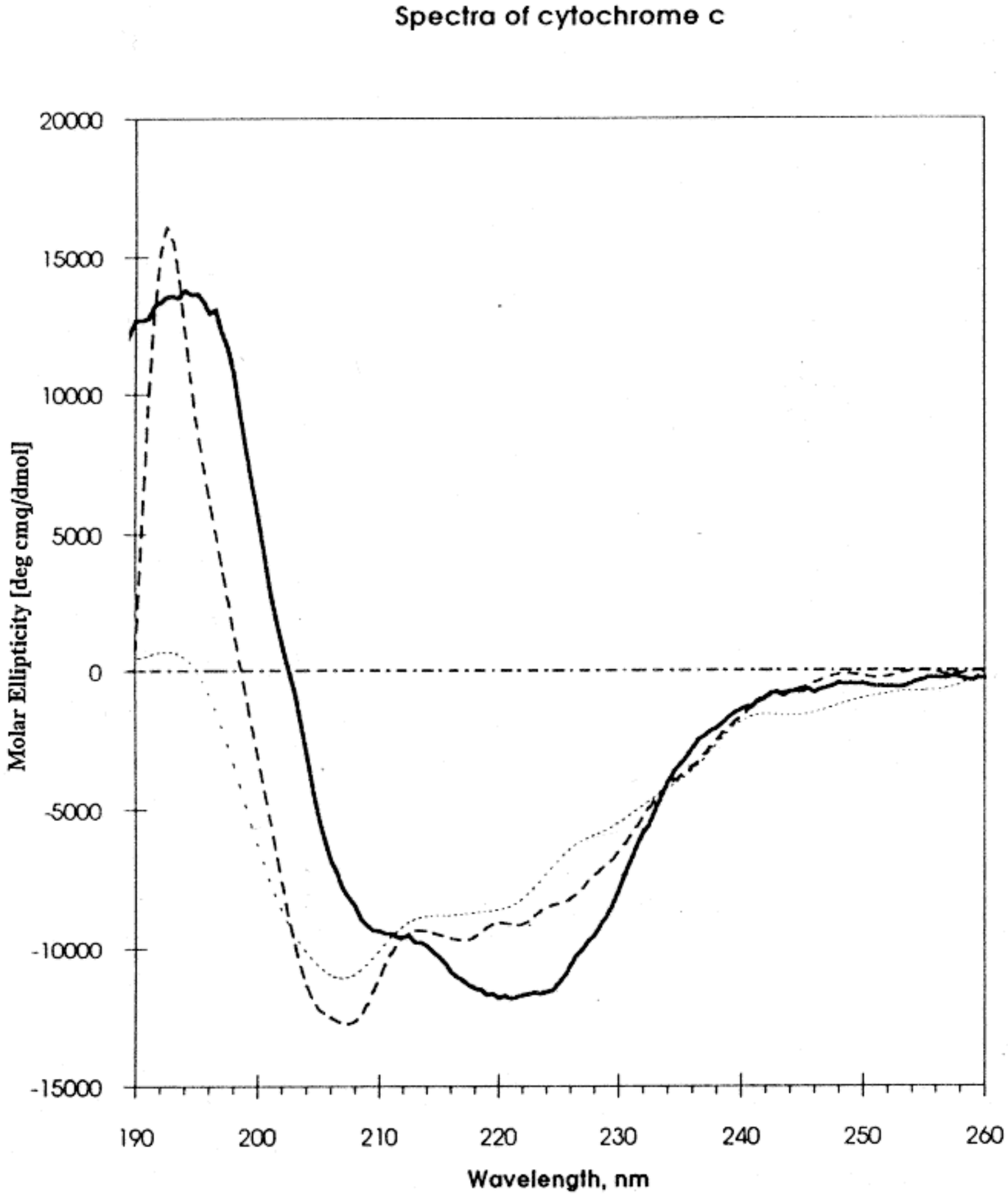

Fig. 5. Circular dichroism spectra of the cytochrome c in phosphate buffer (1), of the solution of reversed micelles (2), of LB films (9 monolayers) deposited by use of the reversed micelles as spreading solution (3), and of LB films (12 monolayers) deposited by use of the AOT reversed micelles without cytochrome c as a spreading solution (4). ——— , cytochrome c in solution (phosphate buffer); – – – , cytochrome c in reversed micelles, hydration degree = 8.6; ·····, Langmuir Blodgett film of cytochrome c, 9 monolayers; –·–·–·–, reversed micelles baseline.

To elucidate the possible structural changes in the protein molecules, circular dichroism spectra were registered (Fig. 5). The first fact observed was a certain change in the CD spectrum of the cytochrome solubilized in reversed micelles with respect to the aqueous solution. This is brought forth by the effect of the micellar matrix, which is known to induce certain conformational changes in proteins [14]. However, it has also been established that such changes do not necessarily imply the loss of functional activity. The spectrum of the protein in LB films, deposited by the present technique, is similar in all respects to that of the protein in reversed micelles. This means that the conformation of the cytochrome c in the LB film is retained as it was before deposition, in a reversed micellar solution. The molar ellipticity values of an "empty" LB film (without protein included), deposited from a solution consisting originally of "empty" reversed micelles of AOT, were used as a baseline for all the measurements to exclude possible effects of adsorption, although it may be of insignificant magnitude. Thus, the spectra presented are only indicative of the protein structure.

The X-ray curve obtained from the sample, containing 10 periods, is presented in Fig. 6. The positions of the Bragg reflections correspond to a spacing of 5.5 nm. The half-width of the reflections corresponds to an order length of about 40 nm, *i.e.* practically the overall film thickness.

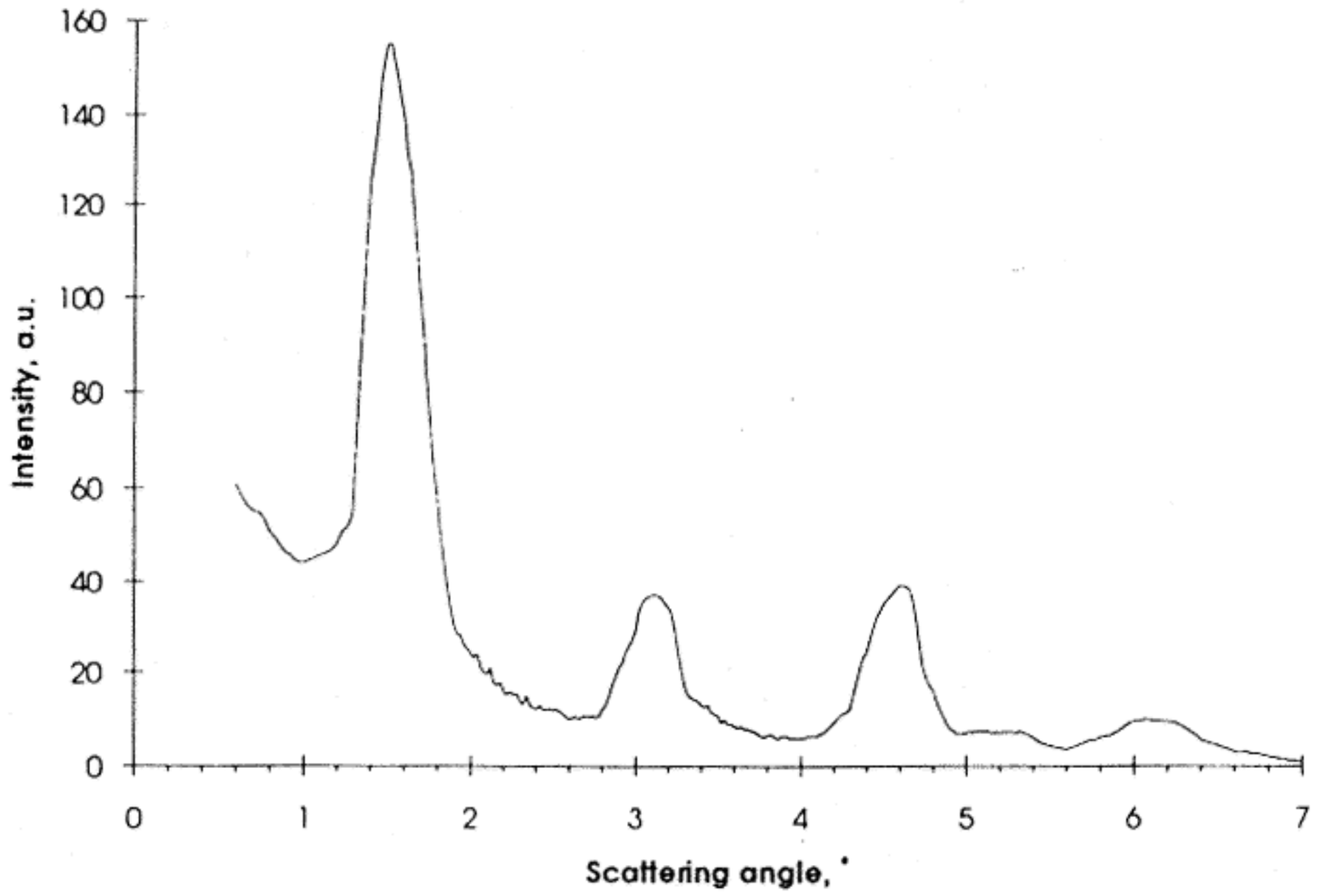

Fig. 6. X-ray pattern of the sample, containing 10 periods (AOT cytochrome c AOT), deposited using reversed micelles as the spreading solution.

4. Discussion

From the above data, as cytochrome c is a water-soluble protein, it appears reasonable to suggest that the structure of the elementary unit of the LB film is that proposed in Fig. 7: a monolayer composed of protein molecules is included between head groups of surfactant monolayers, similar to bilayer or sandwich-type structures in biological membranes. Thus, the period length should represent the thickness of two surfactant monolayers and one protein monolayer.

The cytochrome molecule, as was mentioned before, is approximated by a sphere with a diameter of 3.4 nm. For the period to be equal to 5.5 nm, the surfactant bilayer should have a thickness of about 2.0 nm. The hydrocarbon chain of the AOT molecule contains eight carbon atoms (but only six are arranged in straight line positions). Hence, if the molecules are arranged vertically in the LB films, the length of the surfactant bilayer in the period should be about 1.5 nm, which still gives some space for the dimensions of the head groups, which should not be completely neglected.

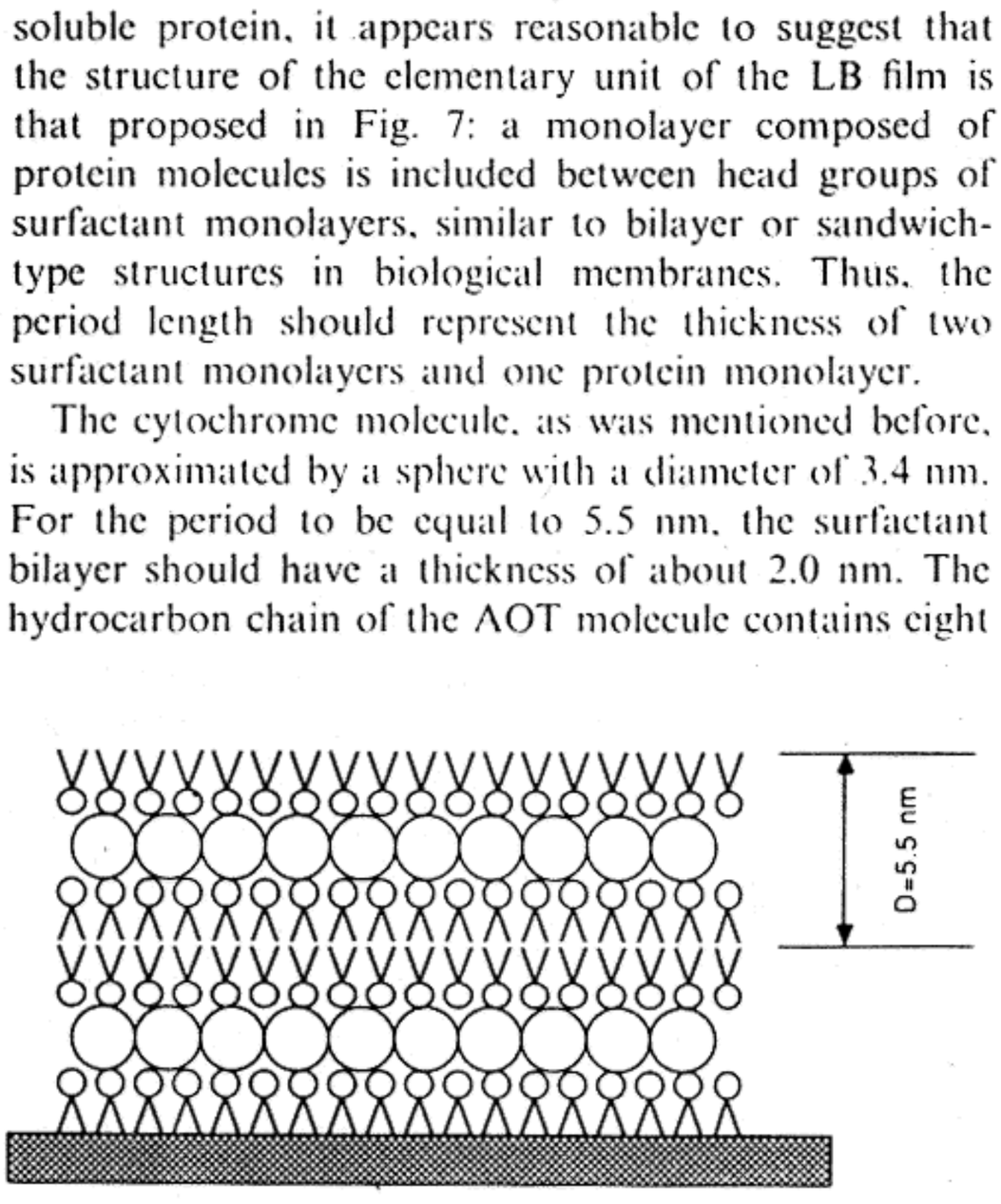

Fig. 7. The model, representing the molecular structure of the LB film, deposited by use of the reversed micelles as spreading solutions. The cytochrome c monolayer is placed between the head groups of AOT molecules in adjacent monolayers.

The following mechanism of metalloprotein LB film formation from reversed micellar solutions can be proposed. After the solution of AOT reversed micelles is placed at the air/water interface, it spreads itself to cover the whole water surface. The solvent evaporates and the micelles in contact with the water rearrange themselves to form a monolayer of surfactant. The protein molecules are below the air/water interface, but associated with the head groups of the surfactant by charge interactions. At the beginning, there is an AOT monolayer with a randomly attached protein monolayer beneath. It is important to have only a monolayer of the protein with no additional protein molecules in the bulk of the subphase (this enables an irregular adsorption of protein molecules to the monolayer to be avoided). Thus, in the resulting LB molecular film, the formation can be conceived as deposition of the protein layers one after the other in a desired variation with other functional elements, all with high reliability and reproducibility.

Repeated utilization of compression–expansion cycles results in a diminishment of the AOT molecular concentration at the water surface. Since the hydrocarbon chain has a short length, the AOT molecules themselves cannot form a stable monolayer at the inter-

face. The surface concentration of AOT molecules decreases because of the penetration of surfactant molecules into the bulk of the subphase during compression (in the form of usual micelles). The reduction of the AOT surface concentration continues until the moment when the amount of AOT molecules is such that the close packing of molecules at the air/water interface corresponds to the close packing of the protein molecules under the water surface. Such a complex monolayer appears to be more stable (which is confirmed by a reproducible $\pi - A$ isotherm and surface potential measurements).

The close packing of the protein in the layer is also substantiated by the good X-ray diffraction pattern that was obtained. If protein molecules in the intermediate layer were not closely packed or not ordered, it would be impossible to obtain such an X-ray curve as that in Fig. 6. The formation of the structure, namely the protein monolayer between surfactant monolayers, is likely to take place during the transition of the substrate through the meniscus, according to the mechanism suggested by Kato [15].

The described procedure of protein LB film formation yielded a highly ordered (with respect to the usual protein LB films) structure, in which the secondary structure of the protein was preserved equal to the structure in the initial solution used for deposition (reversed micelles of Aerosol OT). The fact that protein molecules were intercalated between the surfactant head groups may play an important role, because it might provide a "shield" for the protein molecule against unfavourable external factors. Several applications can be envisaged from such ordered films of metalloproteins, paving in the future the path to bioelectronics [16].

Acknowledgments

The present work was carried out within a contract relative to the National Program of Research on Bioelectronics, given by the Ministry of University and Scientific and Technological Research to the Consorzio Technobiochip, Marciana (LI).

The authors would like to thank Mrs. Kristina Reinfeld for assistance in preparing this manuscript.

References

1 K. B. Blodgett and I. Langmuir, *Phys. Rev.*, *51* (1937) 964.
2 Yu. Lvov, V. Erokhin and S. Zaitsev, *Biol. Membr.*, *4*(9) (1991) 1477–1513.
3 G. Alegria, P. L. Dutton, Z. Popovic and G. L. Kovac, *Biophys. J.*, *47* (1985) 634.
4 D. Tiede, *Biochim. Biophys. Acta*, *811* (1985) 357–379.
5 V. Erokhin, R. Kayushina, Yu. Lvov and L. Feigin, *Studia Biophysica*, *139* (1989) 120–129.
6 V. Erokhin, R. Kayushina, Yu. Lvov and L. Feigin, *Il Nuovo Cimento*, *12D* (1990) 1253.
7 K. Martinek, A. V. Levashov, N. L. Klyachko and I. V. Berezin, *Dokl. Acad. Nauk USSR*, *236* (1977) 920–923.
8 Z. Kozarac, A. Dhathathreyan and D. Mobius, *FEBS Lett.*, *229* (1988) 372.
9 S. A. Darst, M. Ahlers, P. H. Meller, E. W. Kubalek, R. Blankenburg, H. O. Ribi, H. Ringsdorf and R. D. Kornberg, *Biophys. J.*, *59* (1991) 387.
10 Y. Okahata, T. Tsuruta, K. Ijiko and O. Mergura-ku, *Langmuir*, *4* (1988) 1373.
11 L. Belovolova, V. Erokhin and V. Savransky, *Biol. Mem.*, (1993) in press.
12 L. Yu. Mogilevsky, A. T. Dembo, D. I. Svergun and L. A. Feigin, *Crystallographia*, *29* (1984) 587.
13 L. Strayer, *Biochemistry*, Freeman, New York, 1988, p. 470.
14 L. Magid, P. Walde, G. Zampiere, E. Battistel, Q. Peng, E. Trotta, M. Maestro and P. L. Luisi, *Colloids Surfaces*, *30* (1988) 193–207.
15 T. Kato, *Jpn. J. Appl. Phys.*, *27* (1988) 1358.
16 C. Nicolini, M. Adami, F. Antolini, F. Beltram, M. Sartore and S. Vakula, *Physics World*, *5* (1992) 30–34.