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Relation between rheological properties and structural changes in monolayers of model lung surfactant under compression

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Abstract

rSP-C surfactant monolayers spread on a native physiological model substrate show two plateau regions in the π/A-isotherm. The first corresponds to the main phase transition in the monolayer from a LE to a LC phase. Its course is non-horizontal because of the complex composition of the lung surfactant. The second plateau, which is much more pronounced, cannot be attributed to a change of the phase state. Brewster angle microscopy images taken in this region show a sharp apparent decrease of the aggregation degree from the LE to the LC state. This process can be considered as a change in the monolayer orientation relative to the direction of the propagated light. Such a change can be the result of monolayer folding and formation of a thicker layer, which is supported by results of rheological measurements. The dilatation elasticity obtained from oscillating barrier and longitudinal wave measurements reveals a pure elastic behaviour with a steep increase in the second plateau region. Because of the insolubility of the pure lipid components, a possible explanation is squeezing protein components of rSP-C or its complexes with lipids out of the monolayer into the bulk.

Keywords: Lung surfactants; Dilatational viscoelasticity; Brewster angle microscopy; Shear rheology; Oscillating barrier method; Longitudinal wave damping method

1. Introduction

Layers of lung surfactant play a very important role in physiological process such as breathing [1]. Because breathing can be considered as low-frequency oscillations within a relatively narrow frequency range, the viscoelastic properties of such layers strongly influence the parameters of this process. On the other hand, these viscoelastic properties can be closely related to physicochemical processes, taking place in the surface layer during its periodic expansions and compressions in the course of the respiration process and thus give information about the mechanism of this process. Special attention is paid to monolayers at high compression rates, since they can be used as model for natural pulmonary layers in the final stage of the respiratory cycle, when the lung

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alveoli are extremely compressed. Lung surfactants are very complex mixtures comprising components of different surface activity and hydrophobicity. Under periodical changes of the surface area at a high-compressed surface layer state the composition and morphology can also oscillate as a result of the periodic mass exchange processes between this layer and the adjacent bulk phase or within the layer itself. Monolayers composed of components with different surface activity can undergo dramatic transformations. For example, structural changes and accompanying changes of the rheological properties in milk protein layers during displacement by surfactant were recently studied by Mackie et al. [2]. A so-called 'orogenic' mechanism describing this displacement process was proposed. This mechanism includes the gradual heterogeneous accumulation of surfactant in the protein monolayer with subsequent thickening and final complete replacement of protein by the surfactant. Even more similarity to the nature of lung surfactant monolayers exists for β-casein/monoglyceride mixed monolayers as investigated recently by Rodriguez Patino et al. [3]. In such layers the monoglyceride components are hydrophobic and play the same role as the phospholipids in the case of lung surfactant monolayers. It was established that the long-time relaxation phenomena observed in mixed monopalmitin/ and monoolein/ β-casein monolayers could be related to the molecular reorganization of protein molecules during the looping of the amino acid residues into the subphase.

Due to the above-mentioned reasons, the viscoelastic properties of rSP-C surfactant monolayers spread on a subphase have been investigated to model physiological liquids in the living organisms. This rSP-C surfactant was successfully proved for surfactant replacement therapy in animal studies and is studied now in order to demonstrate its efficacy in humans [4]. The chosen frequency range corresponds to the breathing frequency. In order to investigate the viscoelastic properties under dilatation and compression deformations the oscillating barrier and longitudinal wave methods were used. Shear viscosity as a function of molecular area was measured by the torsion pendulum method.

Since the morphology of a monolayer can essentially change during area oscillations due to compression or expansion, the π/A-isotherms of the respective monolayers were simultaneously measured along with a video-recording of Brewster angle microscopy (BAM) images. The comparison of data allows proposing a mechanism for the viscoelastic behaviour of lung surfactant monolayers during the process of periodic deformation in the course of breathing.

2. Materials and methods

2.1. Materials

The subphase on which the rSP-C surfactant monolayers were spread was prepared in the following way. To keep the pH value comparable to those in many biological fluids TRIZMA® 0.05 M buffer solution was chosen as substrate. The composition containing 7.02 g TRIZMA® HCl and 0.67 g TRIZMA® Base dissolved in 1 l water allows to produce solutions of pH 6.91 at 310 K. At lower temperatures the pH slightly increases to pH 7.20 at 298 K. The ionic strength of the subphase was kept constant by addition of 150 mM NaCl. Small admixture of 2 mM Ca^{2+}-ions, another important part of the composition of biological substrates, was added to the subphase.

rSP-C surfactant was obtained from the Byk Gulden, Konstanz, Germany in form of powder, containing 2% recombinant SP-C, mixture of phospholipids dipalmitoylphosphatidylcholine (DPPC) and palmitoyloleoylphosphatidylglycerol (POPG) in a ratio of 70:30, and 5% (w/w) palmitic acid [5–7]. rSP-C is very similar to the human SP-C, except phenylalanine instead of two cysteines in positions 4 and 5, and isoleucine instead of methionine in position 32 [5]. SP-C is relatively small (molecular weight ≈4–5 kD) and highly hydrophobic [4,5].

Methanol (spectroscopic grade from SIGMA) and chloroform (FLUKA) were used as spreading solvents without extra purification. Sodium chloride (Riedel-de-Haen, p.a. purification grade) was heated at 600 °C before use to remove possible surface-active impurities of organic origin. The TRIZMA® Base (purification grade >99.9%) and

TRIZMA® HCl salts (purification grade >99%) used for the preparation of the buffer solution were purchased from SIGMA. $CaCl_2 \cdot 2H_2O$ from FLUKA (for molecular biology, 99.5%) was taken as a source for Ca^{2+}-ions. Milli-Q deionised water with a specific electric conductivity of 18.2 MΩ cm was used to prepare the subphase.

2.2. Methods

Measurements of the π/A-isotherms were performed in a rectangular PTFE Langmuir trough with an area of 518 cm^2. A microbalance with a Wilhelmy plate was used to monitor the surface pressure changes upon monolayer compression, which was performed asymmetrically with a moving barrier at constant velocity of 0.25 cm^2/s. The monolayers were spread from a 10^{-3} M solution of rSP-C surfactant in 1/3 methanol/chloroform, v/v. The amount of solution spread was chosen such a large initial molecular area was established corresponding to the LE state of the monolayer. Recording of the π/A-isotherms was started approximately 10 min after spreading to allow preequilibration of the monolayer. Each measurement was repeated at least three times to reach a satisfactory reproducibility of data.

Evaporation of the subphase during the experiment caused by the very large area of the Langmuir trough can affect the force detector data and the composition of the subphase, especially at temperatures essentially higher than room temperature. To avoid possible artefacts the whole set-up was closed so that a water-saturated atmosphere exists.

The necessary purity grade of the subphase was obtained in the following way. The barrier was quickly moved over the trough surface and subsequently possible surface-active impurities were sucked off. An appropriate grade of purity was reached after some repetitions of this cleaning procedure.

The Langmuir trough was embedded into the BAM device [8] so that the measurement of π/A-isotherms could be carried out along with the video-recording. Single images were obtained subsequently from the videotape via a frame-grabber and were processed by a suitable software [8].

Longitudinal waves were excited by a thin platinum wire (diameter of ≈0.1 mm) oscillating periodically parallel to the liquid surface [9]. Additionally, capillary waves of 160 Hz frequency were generated perpendicular to the direction of longitudinal waves. Longitudinal waves lead to periodic changes of the surface tension and, consequently, low-frequency oscillations of the length of capillary wave. This fact is used to evaluate the length and damping coefficient of longitudinal waves.

If the frequency of the mechanical barrier oscillation along the interface does not exceed 0.2 Hz, the surface tension oscillations along the trough become negligible and the dilatation surface elasticity can be determined by means of the oscillating barrier method. The periodic changes of surface tension can then be detected by the Wilhelmy plate technique. From ratio of surface tension changes to area changes one can evaluate the viscoelastic characteristics of the investigated systems [10,11].

The surface shear rheology was measured with a torsion pendulum rheometer described elsewhere in detail [12]. Basically, a small shear deformation of the liquid surface is produced by a titanium ring, suspended by a tungsten torsion wire to touch the liquid surface. Using this device the interfacial shear elasticity and viscosity of adsorbed and spread surface layers can be determined in a range between 10^{-5}–10^{-6} N/m and 10^{-6}–10^{-3} Ns/m, respectively. For a tungsten wire of 100-μm diameter the oscillation frequency is of the order of 0.1 Hz. All rheological experiments were performed with a deflection angle of 2° so as to minimise distortions of the monolayer structure. The measuring geometry yields an initial relative deformation of the surface of 8.7%. The surface shear rheology of the spread rSP-C surfactant monolayers is measured in dependence of the film pressure. For these studies, a small Langmuir trough made from PTFE is used. The trough consists of two compartments as described in [13] and has a total area of 190 cm^2. The first rheological measurement was performed after 10 min. Then, the rSP-C surfactant monolayer was stepwise compressed by moving the barrier to a definite area. The film pressure was measured using a Wilhelmy plate

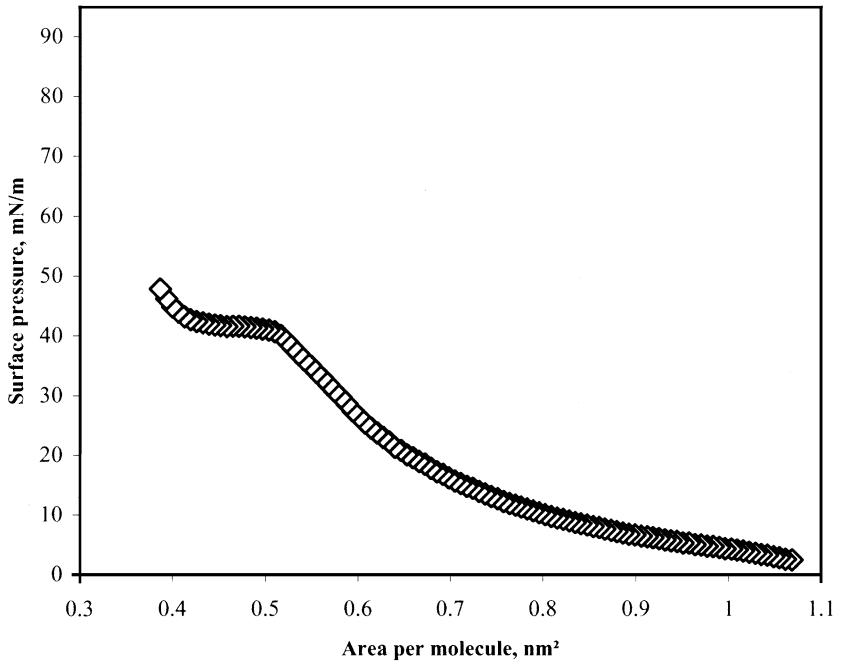

Fig. 1. π/A-isotherm for rSP-C surfactant monolayers spread on the biobuffer subphase.

system (TD 1 from LAUDA/Germany). After each compression step; the rheological experiment, which lasts approximately 30 s, was started after a waiting period of 10 min.

3. Results and discussion

A typical π/A-isotherm for rSP-C surfactant monolayers spread on the biobuffer subphase is shown in Fig. 1. Its shape and position is close to the isotherm characteristics for mixtures of DPPC and 2 mol% SP-C, as reported recently by Wüstneck et al. [14,16]. Although the protein additive is relatively small the whole isotherm is shifted towards larger molecular areas as compared with the π/A-isotherm of pure DPPC [15]. Two regions with distinct changes in the isotherm shape are clearly observed. The first kink point at moderate surface pressures ($\approx$6 mN/m) reveals the onset of a LE–LC phase transition in the monolayer. The second special point lies, on the contrary, in the region of high surface pressures after the first order phase transition is completed. The surface pressure remains practically constant at further compression so that this point can be considered as the onset of a second plateau region, located at 41 mN/m. This can be attributed to the transition of the monolayer to a thicker layer because of squeezing out of the more hydrophilic protein component [16]. This possibility becomes evident from a detailed analysis of π/A-isotherms for the individual components of the mixture. In the maximum compressed state, the minimum area demand of a DPPC molecule is approximately 0.41 nm^2 [17]. The corresponding area for the SP-C molecule in this region of surface pressures is between 2.0 nm^2 [18] and 2.4 nm^2 [16]. In spite of the quite small amount of SP-C in the mixture, the mean molecular area at which the mixed monolayer is in a maximum compressed state should be higher as for the pure lipid. The area at the onset of the second plateau for the rSP-C surfactant (Fig. 1) is approximately 0.5 nm^2 and agrees with the assumption made above. Therefore, only the replacement of the protein component from the monolayer can take place in the molecular areas

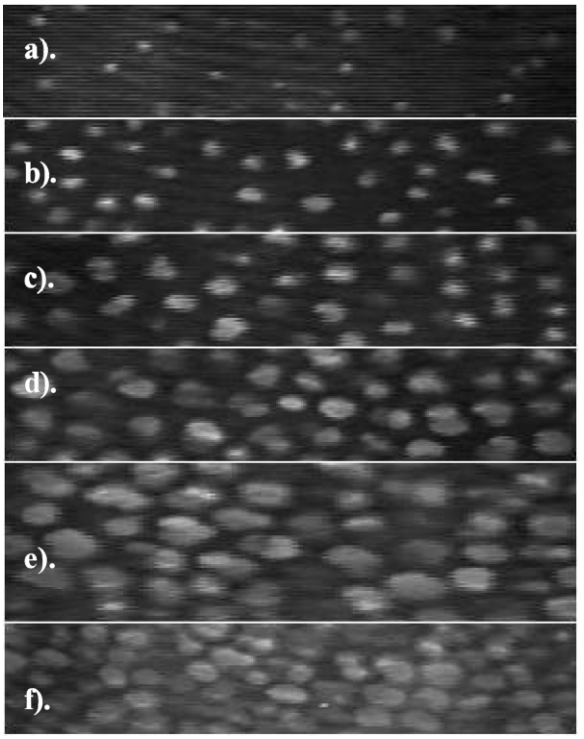

Fig. 2. The BAM images taken simultaneously with the recording of π/A-isotherms given in Fig. 1. The compression grade of monolayer increases in direction: (a) -0.96 nm^2/mol; (b) -0.87 nm^2; (c) -0.77 nm^2; (d) -0.67 nm^2; (e) -0.58 nm^2; (f) -0.48 nm^2. One centimetre on the picture corresponds to approximately 30 μm.

region between 0.5 and 0.4 nm^2. The latter molecular area coincides with the minimum area of the lipid component, so that the whole protein is squeezed out of the monolayer. The BAM images taken simultaneously with recording of the π/A-isotherms are presented in Fig. 2a–f according to the increase in monolayer compression. It can be seen that the start of the LE–LC phase transition is observed exactly at the same area per molecule at which the first kink point in the isotherm is detected. The subsequent growth of domains is accompanied by an essential increase in surface pressure. The non-horizontal plateau region at the LE–LC transition is typical for multicomponent monolayers, according to the Gibbs phase rule. The circular shape of domains indicates strong electrostatic repulsion between the aggregates (Fig. 2d). The part of surface area covered by domains increases gradually upon compression of the monolayer so that the domains begin to contact each other (Fig. 2e). This interaction at a still stronger compression leads to orientational changes in some domains. These domains become grey in the BAM images (Fig. 2f) because of corresponding changes in the polarisation state of reflected light. The quantitative analysis of BAM images leads to the following results. The degree of aggregation, α, increases almost linearly with decreasing molecular area practically until the end of the LE–LC phase transition (Fig. 3). On the contrary, a sharp decrease of this quantity was observed at the attainment of the second plateau on the π/A-isotherm. The same jump can be observed also in dependence of the parameter $(AN_A)^{-1}$ on the degree of aggregation, α. This dependence is usually linear and can be used to evaluate the molecular area in the LC state A_{LC} [8]. The linear fitting yields the slope of the line, which is equal to this area. From Fig. 4 one can see that the parameter A_{LC} has a discontinuity in the region of α, which corresponds to the onset of the second plateau. This phenomenon is apparently caused by changes in the domains orientation as discussed above. Since the whole BAM image is binarised for the evaluation of α, its dark parts were transformed in black and they are attributed to the uncovered surface. Thus, the reoriented domains are counted as part of the monolayer in the LE state. On the other hand, such sharp changes of α and A_{LC} can be considered as an additional independent evidence of the onset of a certain structure change in the monolayer. One of the possible explanations of the described behaviour could be given on the basis of monolayer folding where the protein component of rSP-C surfactant alone or associated with lipids are displaced into the adjacent subphase. A similar statement was already made in [18] where black domains against a bright background were observed in the collapse region of the DPPC/DPPG/SP-C (lipid ratio 8:2 plus 0.4 mol% protein). Such behaviour indicates that the monolayer expands into the subphase creating curved DPPG-enriched protrusions with preferentially incorporated SP-C adjacent to the air–subphase interface. The formation of 3D-struc-

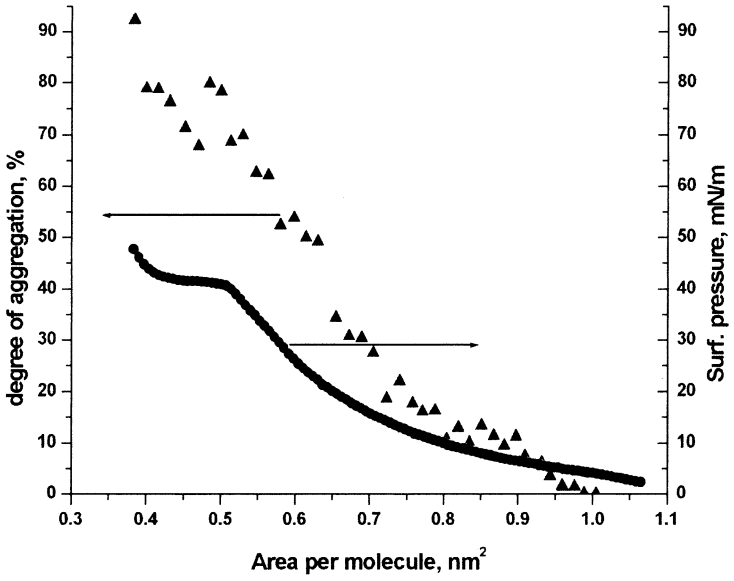

Fig. 3. Degree of aggregation for surfactant monolayer spread on the biobuffer subphase as compared with the corresponding π/A-isotherm.

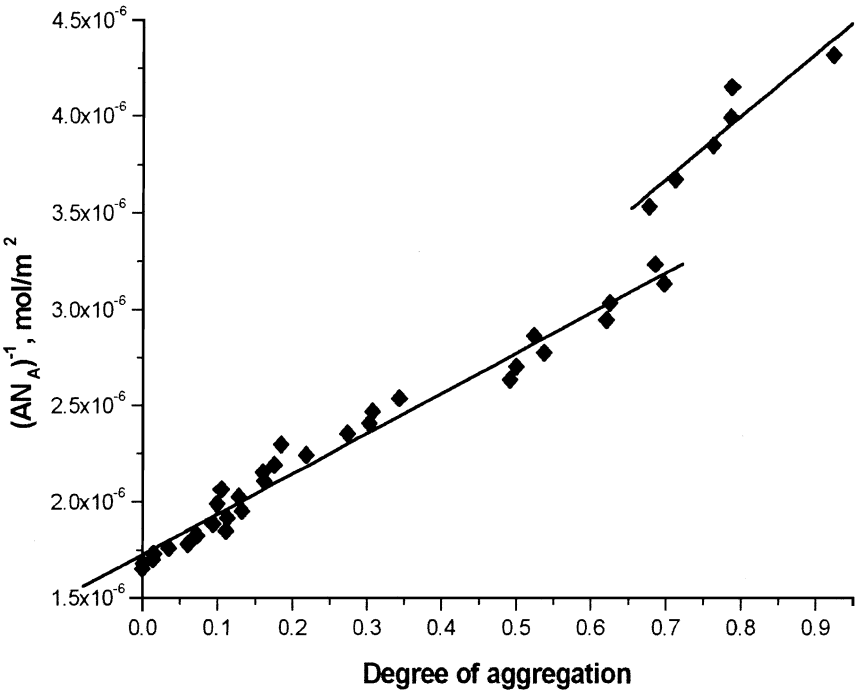

Fig. 4. Discontinuities of the parameters $(AN_A)^{-1}$ and A_{LC} in the region of α corresponding to the onset of the second plateau region of π/A-isotherm.

tures in the DPPC/DPPG/SP-C/B monolayers with the physiologically relevant composition was for the first time directly visualised by Krüger et al. [19] using scattered light microscopy. The softening role of lung proteins and importance of electrostatic interactions in lipid/protein complexes on the formation of 3D-aggregates in the monolayer were also considered in [19]. A thermodynamic model of 3D-surface particle formation in the DPPC/DPPG/SP-C monolayers was proposed recently by Krüger et al. [20]. It was shown that the 3D-aggregate formation is SP-C mediated. The major driving factor is the energy liberated by re-expansion of the initially compressed phospholipid film surrounding the aggregates into the area subsequently depleted from protein. According to this concept, however, the direction in which the 3D-aggregates are penetrated is, on the contrary, opposite to the assumption made in [18]. The helix axis of SP-C together with the associated lipid molecules due to hydrophobic forces are lifted out of the monolayer and inverted into a local bilayer structure extending their hydrophilic headgroups towards the air phase [20]. Effects of SP-B and SP-C additions on the behaviour of 3:1 DPPG:POPG monolayers was reported by Takamoto et al. [21]. In contrast to the mixed monolayer consisting only of phospholipids, the presence of both proteins eliminates the irreversible squeeze-out of the POPG-rich LE phase in the high-pressure plateau region. On the other hand, both SP-B and SP-C induce a 3D-monolayer-to-multilayer transition. Unlike DPPC/DPPG/protein monolayers [18–20], the growing 3D-structures remained in the monolayer plane without detaching from it [21]. The surface pressure at which this plateau occurred is decreased in presence of protein as compared to the pure lipid monolayer. Mechanism of reversible folding was found by Gopal and Lee for the 7:3 DPPC:POPG lipid monolayers [22]. When highly compressed, the monolayer of these lipids buckles into the folds up to several hundred micrometer wide and several millimeters long, even in absence of surfactant proteins. Because the rSP-C surfactant used in the presented work consists mainly of the same components—DPPC, POPG and SP-C, under high compression its monolayers should demonstrate a very similar behaviour as the systems discussed above. This point of view was additionally supported by rheological measurements, both at dilatational and shear deformation. Fig. 5 demonstrates the results obtained from longitudinal waves ($\nu =$ 0.8 Hz) and oscillating barrier ($\nu = 0.2$ Hz) experiments. Both methods lead to similar results for the real and imaginary parts of the complex dynamic elasticity ε_r and ε_i. ε_i was close to zero in the whole range of surface concentrations, and ε_r was independent of frequency. Thus, the behaviour of the monolayer can be described as elastic in the studied frequency range. Such findings agree very well with data obtained by Wüstneck at the same frequencies and surface pressures using the captive bubble technique [14]. At the same time, the ε_r changes are steeper at the attainment of the second plateau and significantly deviate from the static elasticity derived from the π/A-isotherm (solid line on the Fig. 5) indicating strong structural changes. One can assume that extremely high values of ε_r in this region (more than 250 mN/m) originate from a possible transfer of SP-C protein molecules into the subsurface layer, which leads to a solid-like structure of the monolayer consisting only of lipids. Such point of view coincides with the conclusions of Wüstneck et al. [14] about the more elastic character of pure DPPC monolayers in comparison with its mixture with SP-C. Our data are also in the agreement with the results reported by Kretzschmar et al. [23] for monolayers of pure DPPC at $\nu = 0.1$ Hz. The characteristic time of the discussed transfer process should be significantly higher than the period of area oscillations and can, therefore, exert a measurable effect on the elasticity at lower frequencies. For example, the appreciable decrease of ε_r with decreasing oscillations frequency was recently reported in Ref. [14]. The negative values for ε_i at low molar areas A are obviously artefacts and demonstrate the limits of the applied methods. The applicability of the longitudinal wave method is restricted to the region of sufficiently large surface elasticities at low surface coverage. Increase in surface elasticity leads to an increase of the wavelength and decrease of the damping coefficient [24]. When the elasticity modulus approaches 200

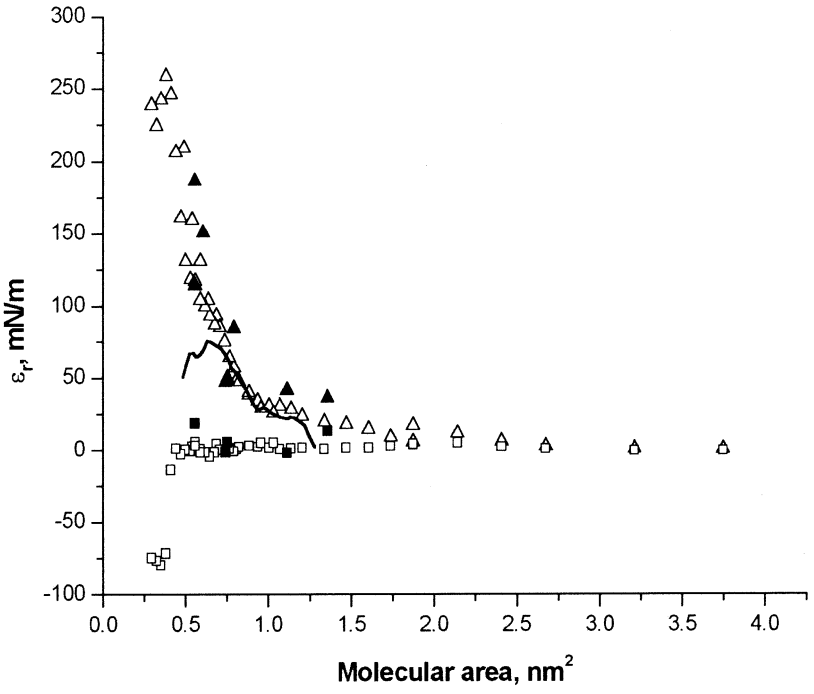

Fig. 5. Real ε_r and imaginary ε_i parts of complex dynamic elasticity of the spread rSP-C surfactant monolayer as functions of area per molecule. Triangles denote real part ε_r, squares—imaginary part ε_i of elasticity, solid line—static value, obtained from π/A-isotherm; filled symbols correspond to $\nu = 0.2$ Hz, open—to $\nu = 0.8$ Hz.

mN/m the wavelength exceeds the length of a Langmuir trough by more than one order of magnitude and the accuracy of measurements decreases strongly. Difficulties in measurements of large dynamic surface elasticities by the longitudinal wave method were also discussed by Maru and Wasan for stearic acid monolayers [25]. However, the results obtained from the oscillating barrier method also confirm the conclusions made from the analysis of BAM images. The discussed structural changes in the rSP-C surfactant monolayers in the high-compressed state were additionally proved by shear rheometry. As in the case of dilatational surface elasticity, the shear viscosity increases steeply when the second plateau region is reached. This experimental finding can be considered as an additional confirmation of the transformation of the rSP-C surfactant monolayer into a thicker and denser layer at high compression.

4. Conclusions

The π/A-isotherms of spread rSP-C surfactant monolayers recorded simultaneously with BAM demonstrate two transition regions. The first can be easily attributed to an ordinary LE–LC 2D-phase transition. Such consideration corresponds with BAM images of LC phase domains visualised in this region. The second plateau region is more pronounced and appears only at very high surface pressures. The corresponding BAM images demonstrate the apparent decrease of the degree of aggregation, α, which is connected with the video image processing algorithm applied. Nevertheless, the discontinuity in the surface concentration dependence of α as well as in the dependence of A_{LC} on α indicates that the monolayer undergoes structural changes in this region. A possible explanation can be given in terms of a squeezing out

of the protein components of rSP-C surfactant into the adjacent bulk phase, which leads to thickening and to the formation of a more elastic monolayer. This assumption agrees well with the results of dilatation and shear rheology measurements. The dilatational dynamic elasticity and shear viscosity increase drastically at the attainment of the second plateau in the π/A-isotherm.

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