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Dynamic Properties of Adsorption Layers of κ-Casein Fibrils

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Cite This: https://doi.org/10.1021/acs.langmuir.3c01950 Read Online

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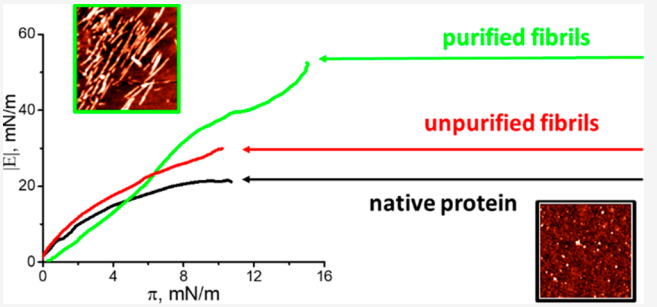
ABSTRACT: The dynamic surface properties of native κ-casein solutions and aqueous dispersions of its fibrils differ significantly from the corresponding properties of the systems with globular proteins. The dependence of the dynamic surface elasticity of κ-casein solutions on surface pressure has a local maximum, indicating partial displacement of macromolecules from the proximal region of the surface layer to the distal one. This dependence becomes monotonic for fibril dispersions, similar to the results for dispersions of globular protein fibrils, but unlike the latter case, the surface elasticity close to the steady state reaches values that are approximately four times higher than the data for native protein solutions at the same concentrations.



INTRODUCTION

Amyloid fibrils play a key role in the development of neurodegenerative diseases[1] such as Alzheimer's disease,[2,3] Parkinson's disease,[3,4] prion diseases,[5,6] and Huntington's disease.[7] At the same time, some amyloids can play an important role in various living organisms; for example, the human premelanosome amyloid fibrils protect melanocytes from damage in the course of melanin synthesis.[8]

Recently fibrillar aggregates have been frequently used to stabilize foams and emulsions in food, cosmetic, and pharmaceutical industries.[9−11] New materials on the basis of protein fibrils find various applications due to their unique properties.[9,12−17]

Dynamic surface tension and dilatational surface elasticity are key properties determining the stability of foams and emulsions. The adsorption layers of amyloid fibrils are characterized by the higher elasticity than layers of native proteins.[18−23] Moreover, the fibrils can provide multifunctionality to emulsions due to their thermal stability and antioxidant activity.[24−26]

κ-Casein is one of the main milk proteins and takes part in the formation of casein micelles. Unlike most of water-soluble proteins, it has only insignificant elements of tertiary structure in aqueous solutions.[27] The molecular weight of this protein is about 19 kDa, and it consists of 169 amino acids, among which are two cysteine residues: 11Cys and 88Cys. In aqueous solutions κ-casein exists in the form of oligomers (up to decamers), which are in equilibrium with monomers.[28] Oligomers are formed by disulfide bonds between cysteine residues in random combinations: 11Cys−11Cys, 88Cys−88Cys, and 11Cys−88Cys.[29] Due to electrostatic and hydrophobic interactions, oligomers spontaneously form spherical aggregates with a radius of about 10 nm.[30] κ-Casein forms fibrillar aggregates at physiological pH and at temperatures of 36 °C and above.[31−35] The fibril formation can be accelerated by the addition of a reducing agent (e.g., dithiothreitol and β-mercaptoethanol).[31,32,34] Besides, the fibril formation of κ-casein is possible at acidic conditions at pH = 2.0 and 90 °C.[36] The mechanism of κ-casein fibril formation proposed by Ecroyd et al. is based on the antiparallel β-sheets stacking of monomeric protein molecules.[33] The addition of reducing agents accelerates the formation of aggregates by increasing the monomer concentration.

The information on the surface properties of κ-casein solutions is quite scarce,[37,38] and the surface properties of dispersions of its fibrillar aggregates have not been studied yet to the best of our knowledge. Note that measurements of the surface properties of fibril dispersions usually encounter serious difficulties due to the strong influence of impurities of high surface activity. The protein hydrolysis at elevated temperatures results in formation of peptides of low molecular weight and high surface activity.[39] They are adsorbed fast and strongly influence the surface properties. A similar problem appears in the course of investigation of the surface properties of dispersions of spherical protein aggregates.[29,40−43] It has been shown recently that multiple centrifugation of the dispersions of protein spherulites gives a possibility to purify

Received: July 13, 2023
Revised: October 9, 2023
Accepted: October 9, 2023

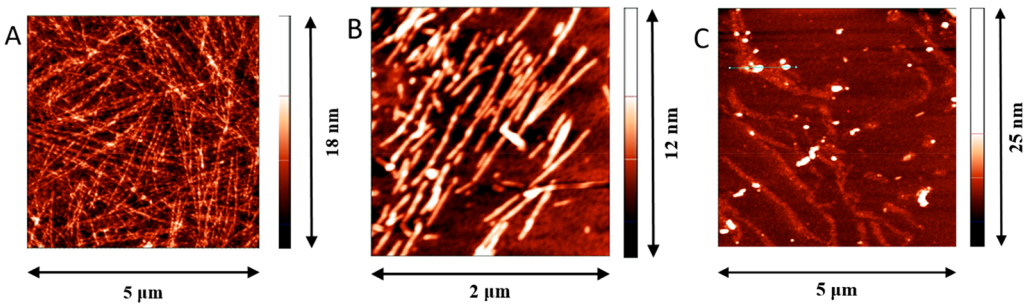


Figure 1. AFM images of κ-casein fibrils dispersions after preparation (A); adsorption layer of one time purified dispersion before compression (B) and after compression (C) with a concentration of 0.002 mg/mL at pH 7.0.

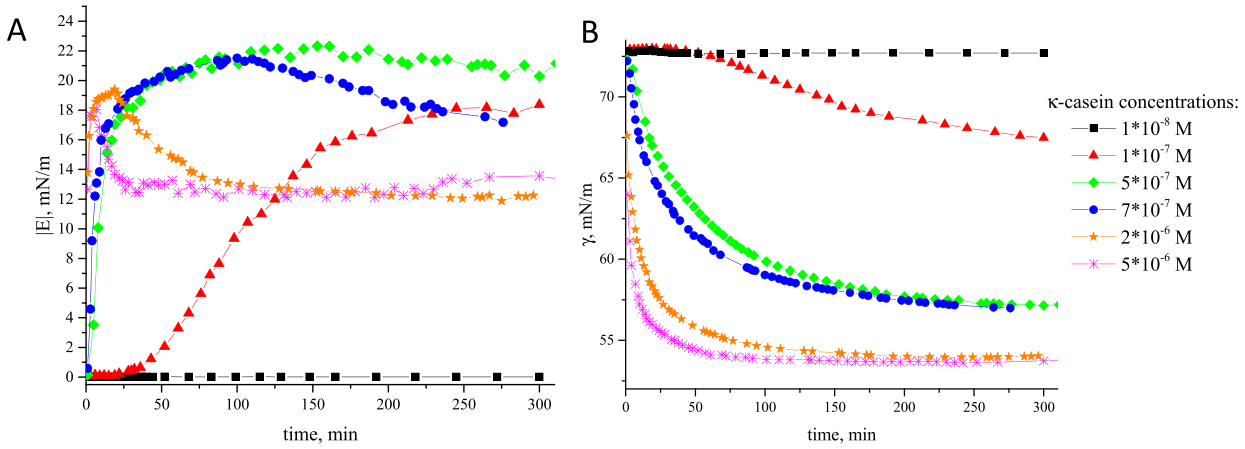


Figure 2. Kinetic dependencies of the modulus of the dynamic surface elasticity (A) and dynamic surface tension (B) of native κ-casein solutions at pH 7.0 with concentrations of 1×10^{-8} M (black squares), 1×10^{-7} M (red triangles up), 5×10^{-7} M (green diamonds), 7×10^{-7} M (blue circles), 2×10^{-6} M (orange asterisks), and 5×10^{-6} M (magenta snowflakes).

them from the impurities.[40,43] The same purification method was also applied to dispersions of β-lactoglobulin and lysozyme fibrils and allowed reducing the influence of peptides of low molecular weight on the properties of spread fibril layers.[21]

The aim of this work is to apply the same purification method to the dispersions of κ-casein fibrils, to determine their dynamic surface properties, and to compare them with the surface properties of native protein solutions and of the aqueous dispersions of purified fibrils of other proteins. Another aim consists of the determination of the dynamic surface properties of κ-casein solutions and in their comparison with the surface properties of globular protein solutions.

EXPERIMENTAL SECTION

Materials. κ-Casein ($M_w \approx 19000$ Da, Sigma-Aldrich) was used as received. Protein aqueous solutions were prepared from the stock solution that was stored in a refrigerator for no more than 7 days at a temperature of 4 °C. NaH_2PO_4 and Na_2HPO_4 (Sigma-Aldrich, Germany) were used to prepare buffer solution with an ionic strength of 0.02 M and pH 7. Triply distilled water was used to prepare all of the solutions. Sodium chloride (Vekton, Russia) was heated in an oven at 750 °C to remove organic impurities.

κ-Casein solution with a concentration of 5 wt % was dialyzed against HCl solution (pH = 2.0) for 3 days. The final concentration was about 1 wt %. The dialyzed solution was centrifuged (10000*g*, 20 min) and filtered through a membrane with pore sizes of 200 nm (Vladipor, Russia). The fibrils were prepared by heating the protein solution at a temperature of 90 °C for 24 h as described by Pan et al.[36] The obtained fibril dispersions were used for 20 days after preparation and stored in a refrigerator at 4 °C.

The final protein concentration in dispersions was determined by a gravimetric method. A given volume of the fibril dispersion was dried at 60 °C, and the remaining dry material was weighted.

The application of the atomic force microscopy (AFM) showed that the formed κ-casein fibrils are almost straight, thin, and long rods with a height of about 3−4 nm and a length of a few micrometers (Figures 1A and S1).

The fibril purification from the impurities of low molecular weight was performed by successive centrifugations (10000*g*, 40 min), replacement of the supernatant with triple distilled water, and redispersion with shaking. The degree of purification was characterized as the number of centrifugation cycles and the subsequent replacement of the supernatant.

Methods. The surface tension was measured by the Wilhelmy plate method using a paper plate with an accuracy of ±0.2 mN/m. The dynamic dilatational surface elasticity was determined by the oscillating barrier method.[44] This method is based on sinusoidal oscillations of the solution surface area and measurements of the induced oscillations of the surface tension. The measurements were performed using the ISR instrument (KSV-NIMA, Finland, Sweden). In the case of small oscillations the dynamic surface elasticity is reduced to the ratio of the complex amplitudes of the surface tension oscillations $\Delta\gamma$ and the relative surface area $\Delta A/A$.

$$\varepsilon = \frac{\Delta\gamma}{\Delta A/A}$$

The real ε_R and imaginary part ε_{Im} of the dynamic surface elasticity can be calculated from phase shift φ between the surface tension and surface area oscillations:

$$\varepsilon = \varepsilon_R + i\varepsilon_{Im} = \left|\frac{\Delta\gamma}{\Delta A/A}\right|(\cos\varphi + i\sin\varphi)$$

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For the investigated layers of native κ-casein and its fibrils, the imaginary part of dynamic dilatational surface elasticity was much less that the real part. Therefore, only the modulus of the dynamic surface elasticity is discussed below. During all the measurements, the oscillation frequency was 0.05 Hz and the relative surface area change was 5%.

AFM (NT-MDT microscope, Russia) in a semicontact mode was used to determine the layer micromorphology. The layers were deposited on a freshly cleaved mica plate by the Langmuir− Schaefer method and dried after that in a desiccator for several days at room temperature.

The Brewster angle microscopy provided information on the macroscopic and mesoscopic morphology of the adsorption layer at the air−water interface. A BAM 1 instrument (Nanofilm Technology, Germany) equipped with a 10 mV He−Ne laser was used for this purpose.

RESULTS AND DISCUSSION

Dynamic Surface Properties of Native κ-Casein Solutions. The dynamic surface elasticity (Figure 2A) and surface tension (Figure 2B) of κ-casein solutions were measured as a function of surface age and protein concentration (from 1×10^{-8} to 5×10^{-6} M). At concentrations lower than 1×10^{-7} M κ-casein does not demonstrate a noticeable surface activity. The surface tension and dynamic surface elasticity were close to the corresponding values for pure solvent. At a concentration of 1×10^{-7} M one could observe an induction period of about 30 min, after which the dynamic surface elasticity and surface pressure started to increase (Figure 2). A similar induction period of the kinetic dependences of surface properties has been previously observed for solutions of some other proteins and is connected with the peculiarities of the equation of state of the adsorption layer.[45−47] Some time is needed to obtain surface concentrations that affect the surface properties. The rate of change of the dynamic surface elasticity and surface pressure increases with an increase of κ-casein concentration. In the concentration range 1×10^{-7}−7×10^{-7} M the dynamic surface elasticity and surface pressure monotonically increase with the surface age. A local maximum of the kinetic dependencies of the dynamic surface elasticity appears at a further increase of the protein concentration. At concentrations of 2×10^{-6} and 5×10^{-6} M the dynamic surface elasticity and surface tension approach steady-state values of 12 and 54 mN/m.

If the dynamic surface elasticity is plotted as a function of the surface pressure, the dependences at different concentrations almost coincide (Figure 3). This means that the distinctions of the curves in Figure 2 are mainly caused by the differences in the rate of protein transport from the bulk phase to the interface, and the equilibrium equation of state holds for the adsorption layer independently of the adsorption time for all the concentrations studied.

At relatively low protein concentrations, the surface pressure corresponding to the local maximum, ∼14 mN/m, was not obtained in the course of measurements. The local maximum indicates a conformational transition in the surface layer. Similar behavior was observed previously for another nonglobular protein, β-casein.[48,49] The structure of β-casein has some distinctions from that of κ-casein.[50] β-Casein is the most hydrophobic among the casein fractions, although it has a strongly charged N-terminal region. As a rough physical model of β-casein, one can consider a diblock copolymer with relatively hydrophilic and hydrophobic blocks. N-terminus residues 1−40 of β-casein contain all the net charge of the molecule, whereas the C-terminal section (residues 136−209) contains many the apolar residues and is characterized by a small charge and high hydrophobicity. For κ-casein, there is no such strong division into polar and nonpolar parts. Negative charges occur only in the N-terminal fragment of residues 1−20 and in the C-terminal fragment of residues 115−169. Positive charges are found only in the N-terminal segment. Most hydrophobic patches are also found in this segment, between residues 21 and 110; segments 1−20 and 110−169 show predominantly hydrophilic behavior. The presence of residues Cys11 and Cys88 creates a complex disulfide bonding pattern among κ-casein molecules. As a result, β-casein and κ-casein cannot be modeled by a linear block copolymer.

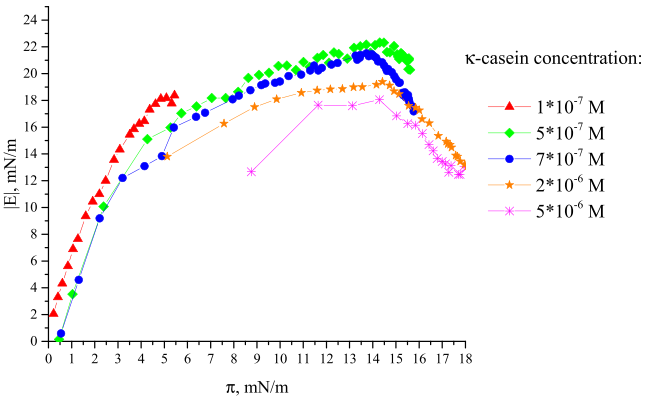

Figure 3. Modulus of the dynamic surface elasticity as a function of the surface pressure of native κ-casein solutions at pH 7.0 with concentrations of 1×10^{-7} M (red triangles up), 5×10^{-7} M (green diamonds), 7×10^{-7} M (blue circles), 2×10^{-6} M (orange asterisks) and 5×10^{-6} M (magenta snowflakes).

The dynamic surface elasticity of β-casein solutions has two maxima corresponding to the displacement of the relatively hydrophilic and relatively hydrophobic parts of the molecule to the distal region of a surface layer in the form of loops and tails.[51,52] The distribution of charged and hydrophobic groups in a κ-casein molecule is more homogeneous, and as a result only one maximum at a surface pressure of 14 mN/m is observed similar to the case of solutions of unfolded globular proteins.[46,53,54] It is possible to assume that flexible κ-casein molecules are transferred to the surface layer as trains without long loops and tails in the course of the initial adsorption step (Figure 4). The further adsorption results in an increase of the surface pressure, and when it reaches 14 mN/m, some parts of

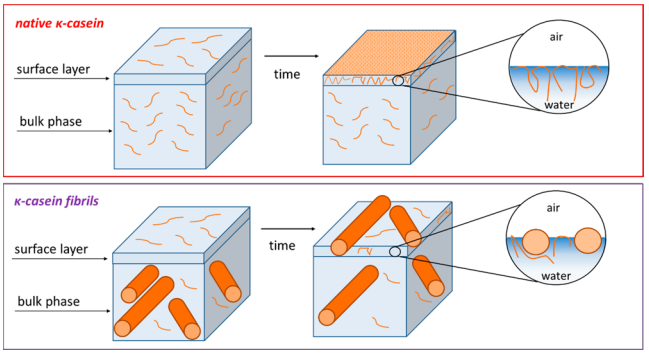

Figure 4. Scheme of adsorption layers of native κ-casein and its fibrillar aggregates with increasing surface pressure and surface age.

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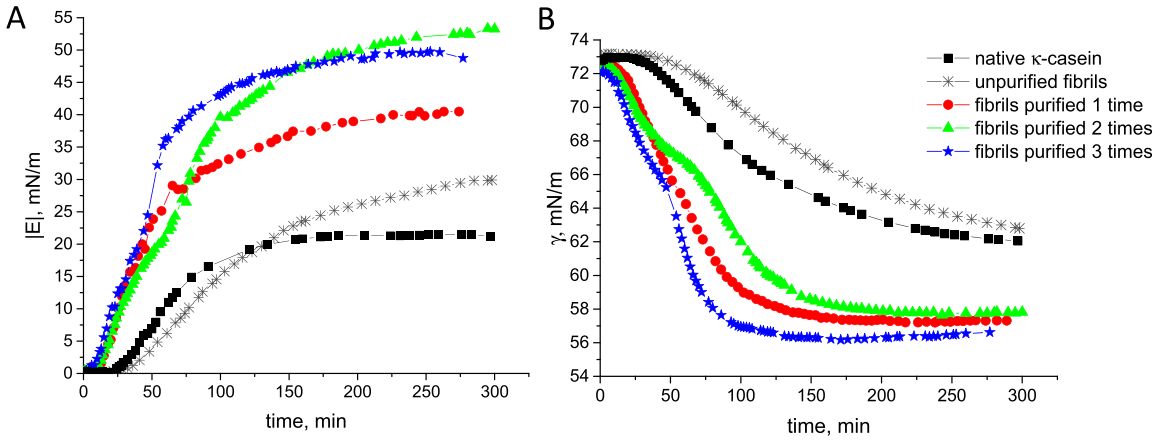

Figure 5. Kinetic dependencies of the modulus of the dynamic surface elasticity (A) and surface tension (B) of native κ-casein solution with a concentration of 1×10^{-7} M (black squares) and of κ-casein fibril dispersions with different degrees of purification: unpurified (gray snowflakes), purified 1 time (red circles), purified 2 times (green triangles up), and purified 3 times (blue asterisks) with a concentration of 0.002 mg/mL at pH 7.0 and with 0.1 M NaCl.

the macromolecule are displaced from the proximal region of the surface layer to the distal one.

The addition of 0.1 M NaCl to solutions of κ-casein decreases the electrostatic adsorption barrier, and the rate of changes of the dynamic surface properties increases (Figure S2).

Dynamic Surface Properties of κ-Casein Fibril Dispersions. Fibrils were obtained from concentrated κ-casein solutions (about 1 wt %) at acidic environment by heating at 90 °C for 24 h. The obtained aggregates (Figure 1A) represent long almost straight threads with the height of about 3−5 nm and the length of a few micrometers (Figure S1). One can see a periodical increase of the fibril height with a period of about 100 nm, which could be a consequence of twisted ribbon shape of an aggregate.[55,56] After fibril preparation, the dispersion can contain unreacted protein molecules, hydrolyzed peptides, and aggregates of small size. The peptides have a relatively low molecular weight and presumably higher surface activity than the fibrils and influence strongly the dynamic surface properties. Therefore, careful purification of the samples is required. The previous results for various aggregates of lysozyme and β-lactoglobulin (BLG) show that the use of unpurified dispersions leads to the dynamic surface properties close to the data for native protein solutions.[21,29,40−43] On the contrary, the properties of the adsorption layers of κ-casein fibrils differ from those of native protein layers even in the case of unpurified aggregates (Figure 5). The increase of the surface elasticity from about 22 mN/m for native κ-casein solutions to about 30 mN/m for unpurified dispersion is presumably a result of the joint adsorption of unreacted protein molecules together with protein aggregates. After an initial adsorption step corresponding mainly to the admixture adsorption, the dynamic surface elasticity changes slower than the surface tension. At the same time, the dynamic surface elasticity exceeds the values for pure protein solutions already at the beginning of measurements and increases with surface age at an almost constant surface pressure. It is possible to assume that the adsorption of admixtures is accompanied by the adsorption of fibrils, leading to a more rigid adsorption layer and to an increase of the surface elasticity.

The effect of fibrils becomes stronger for purified dispersions when the relative concentration of fibrils increases. The adsorption layer becomes more rigid, and the dynamic surface elasticity reaches 52 mN/m. All the kinetic dependences for fibril dispersions are monotonic. The significant increase of the dynamic surface elasticity of κ-casein fibril dispersions, as compared with solutions of the native protein, distinguishes this system from previously studied dispersions of BLG and lysozyme fibrils.[21] In the latter case, the absolute values of the dynamic surface elasticity were higher (up to 60 mN/m for BLG and 80 mN/m for lysozyme fibril dispersions). At the same time, the obtained results were rather close to those for pure protein solutions.

Another peculiarity of the kinetic dependencies of the dynamic surface elasticity and surface tension for κ-casein fibril dispersions is an inflection point at a surface pressure close to 7 mN/m and a surface elasticity close to 22 mN/m. Presumably even a thorough cleaning does not allow removal of all the admixtures. The unreacted protein and polypeptides of high surface activity and relatively low molecular weight are adsorbed first at the air−water interface (Figure 4). This idea is supported by the dependences of the dynamic surface elasticity on surface pressure, which are close to each other for fibril dispersions and pure protein solutions at surface pressures below 7 mN/m (Figure 6). At higher surface pressures the dynamic surface elasticity increases with the increase of the number of purification steps and is determined mainly by the adsorption of fibrils. The change of the mechanism of the adsorption layer formation at surface pressures close to 7 mN/m results in inflection points in Figure 5. The increase of the solution ionic strength also influences the adsorption kinetics due to a decrease in the electrostatic adsorption barrier (Figure S3).

The increase of dispersion concentration increases the rate of changes in the dynamic surface properties (Figure S4). In this case the increase of concentrations of both fibrils and admixtures are responsible for the observed changes of the kinetic dependencies of the surface properties.

The change of the surface layer structure with surface age and the increase of surface pressure can be illustrated by AFM images for the layers transferred onto the mica surface by the Langmuir−Schaefer technique. At early steps of adsorption and low surface pressures (2 mN/m, Figure S5A) one can see an almost clear surface with a small number of aggregates. Their heights do not exceed 2 nm. At surface pressures about 13 mN/m and at the increase of surface age one can observe a

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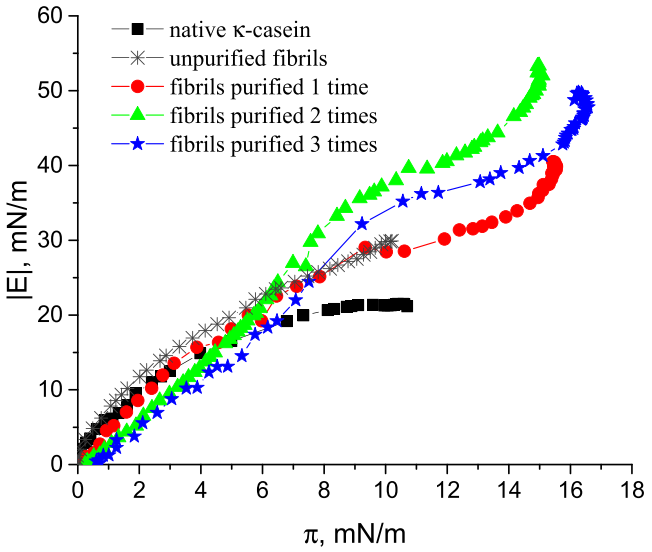

Figure 6. Modulus of the dynamic surface elasticity as a function of the surface pressure of native κ-casein solution with a concentration of 1×10^{-7} M (black squares) and κ-casein fibrils dispersions with different degree of purification: unpurified (gray snowflakes), purified 1 time (red circles), purified 2 times (green triangles up), and purified 3 times (blue asterisks) with a concentration of 0.002 mg/mL at pH 7.0 and with 0.1 M NaCl.

layer with a larger number of aggregates of different sizes (Figure S5B). Finally, the fibrils can be observed in the adsorption layer at high surface pressures (Figure 1B). The rod-shaped aggregates in this figure look shorter than the fibrils in the bulk phase (Figure 1A). This is possible if the aggregates are visible only partly, and they are partly immersed in a layer of other components. On the other hand, the small aggregates can be adsorbed faster than the longer ones due to their higher diffusion coefficient.

AFM images after compression show some relatively thick and rather vague bands, but neither rodlike aggregates nor threadlike fibrils are visible (Figure 1C). The BAM images can also show the macroscopic heterogeneity of the protein adsorption layer. One can see some stripes appear after the layer compression (Figure 7A). A mechanical disturbance by a thin rod leaves a trail in the layer, disappearing for a few minutes and indicating the layer softness (Figure 7B).

CONCLUSIONS

The local maximum and low steady-state values of the dynamic surface elasticity of κ-casein solutions are typical for solutions of nonglobular proteins and linear flexible amphiphilic polymers. The local maximum is connected with the formation of a distal region of the surface layer as a result of the displacement of some parts of κ-casein molecules in the form of tails and loops from the proximal region of the surface layer. The comparison of the dilatational viscoelasticity of κ-casein adsorption layers with that of β-casein layers indicates a more uniform distribution of hydrophobic and hydrophilic amino acid residues along the protein chain in the first case. This results in a single maximum of the surface elasticity instead of two local maxima in the latter case. The dynamic surface properties of aqueous dispersions of κ-casein fibrils differ significantly from the properties of native protein solutions, unlike the surface properties of dispersions of globular protein fibrils. In particular, the dynamic surface elasticity of κ-casein fibril dispersions is about two times higher than that of native protein solutions. The inflection point of the dependences of the dynamic surface elasticity and dynamic surface tension can be connected to two steps of the adsorption layer formation. The first step corresponds mainly to the adsorption of polypeptides of low molecular weight peptides, while the second one is the adsorption of fibrils.

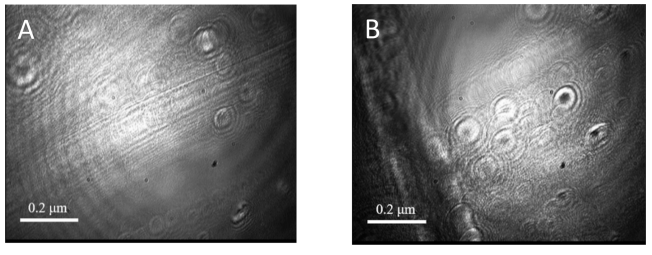

Figure 7. BAM images for adsorption films of unpurified κ-casein fibrils dispersions at pH 7.0 after compression to 80% of the initial surface area (A) and after mechanical disturbance with thin needle (B).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.langmuir.3c01950.

Figure S1: AFM images of κ-casein fibrils dispersions after preparation (A, B). Cross-section profile (C) corresponds to the blue line on image B; Figure S2: kinetic dependencies of the modulus of the dynamic surface elasticity (A) and the dynamic surface tension (B) of native κ-casein solutions with concentrations of 1×10^{-7} M (red squares) and 1×10^{-6} M (blue circles) in phosphate buffer at pH 7.0 (closed symbols) and with 0.1 M NaCl (open symbols); Figure S3: kinetic dependencies of the modulus of the dynamic surface elasticity (A), the dynamic surface tension (B), and the dynamic surface elasticity versus surface pressure (C) of 2 times purified κ-casein fibrils dispersions at pH 7.0 and different ionic strength; Figure S4: kinetic dependencies of the modulus of the dynamic surface elasticity (A) and the dynamic surface tension (B) and the dynamic surface elasticity versus surface pressure (C) of 2 times purified κ-casein fibrils dispersions at pH 7.0 and 0.1 M NaCl with various dispersion concentrations; Figure S5: AFM images of κ-casein fibrils adsorption layer obtained at different surface pressures: 2 mN/m (A) and 13 mN/m (B); the dispersion concentration was 0.002 mg/mL, pH 7.0 (PDF)

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https://doi.org/10.1021/acs.langmuir.3c01950
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Author Contributions

O.Y.M.: writing−original draft, investigation, conceptualization; A.V.A.: validation, investigation; A.G.B.: methodology, investigation; G.L.: conceptualization, writing−review and editing; R.M.: conceptualization, writing−review and editing; I.P.: conceptualization, writing−review and editing; A.R.R.: investigation, writing−original draft, methodology; B.A.N.: methodology, conceptualization, writing−original draft, supervision.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was performed in commemoration of the 300th anniversary of St. Petersburg State University's founding. The work was financially supported by Russian Science Foundation (project no. 21-13-00039). The authors are also grateful to the resource centers of SPbU (Centre for Optical and Laser Materials Research, the Chemical Analysis and Materials Research Centre, Centre for Diagnostics of Functional Materials for Medicine, Pharmacology and Nanoelectronics, Interdisciplinary Resource Centre for Nanotechnology, Centre for Microscopy and Microanalysis, Centre for Molecular and Cell Technologies and Centre for Innovative Technologies of Composite Nanomaterials) for the use of their equipment.

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https://doi.org/10.1021/acs.langmuir.3c01950
Langmuir XXXX, XXX, XXX−XXX