Octamers participation in the formation of lysozyme ordered layers from crystallization solutions

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Abstract. The results of the study of thin film ordered protein structures formed from polydisperse solutions of lysozyme using Langmuir technology are presented. The proposed method for producing protein films is based on a modification of the Langmuir-Schaeffer method, which consists in using a pre-prepared protein solution with the addition of a precipitant. The pre-prepared protein solutions’ parameters (protein and precipitant concentrations, buffer type, etc.) correspond to protein crystallization conditions. It is assumed that protein oligomers formed in the solution as a result of the addition of the precipitant (in particular, for lysozyme these oligomers include octamers) are directly involved in the formation of Langmuir protein layers on the surface of the liquid and on solid substrates. Using the method of grazing-incidence X-ray standing waves, the structure of multilayered protein systems formed from polydisperse solutions was studied, which made it possible to determine the position of precipitant ions (NaCl, CuCl₂ and NiCl₂) relative to the protein layer. The method of processing the X-ray reflectivity and grazing-incidence X-ray standing waves data, based on the use of information on the atomic structure of lysozyme octamers isolated from the crystal lattice, made it possible to determine the thickness and electron density of protein films and to reveal the orientation of protein molecules in the layer.

1. Introduction

Nowadays, there are successfully designed and implemented system devices where proteins on substrates are used as functional units in biosensors. In these biosensors, protein molecules are organized as a layered ensemble on a substrate which is used in scientific and industrial research field. Such layer organization of the molecules allows one to increase the contact area between the biosensor matrix (an electrode on a solid substrate) and protein molecules [1]. Earlier for the low weight molecular organic compounds, it was shown that using the ordered polymer films for several photovoltaic materials creation can significantly increase the efficiency of these structures compared with that one obtained from disordered materials [2]. The efficiency of biosensor devices is suggested to be improved by using ordered protein films. At this stage, the aim is to orient the protein molecules in the required way, which is especially important when we use enzymes as a functional element of the sensor, since the correct orientation of the catalytically active enzyme center directly affects the functional properties of the created device.
To obtain layered systems based on organic molecules, the Langmuir technology is currently widely used. This technology usually involves the use of a monodisperse solution of molecules, what can lead to the formation of films with an unknown structure [3-5]. Previously, using small-angle x-ray and neutron scattering methods, it was shown that in the solution of lysozyme with the addition of a sodium chloride precipitant (NaCl) under the conditions of tetragonal lysozyme crystal growth, oligomeric fractions of lysozyme are formed. These oligomers include octamers, which can be elementary growth units of the future lysozyme tetragonal crystal [6 -10]. Based on this fact, a (fundamentally new) method was developed for the formation of lysozyme films on solid substrates using a modification of the Langmuir-Schaeffer. For this we used the protein crystallization solution with the addition of NaCl instead of monodisperse solution of protein [11]. A study of the structure of such films by X-ray reflectivity (XRR) and grazing-incidence X-ray standing waves (GI-XSW) (or X-ray standing waves method under total external reflection [XSW-TER] conditions) showed that the thickness of the films is comparable to the size of the lysozyme octamer, and that Cl – precipitator ions form a thin layer about 1 nm thick at the air/film interface. However, the question of the orientation of octamers in the protein film remains unclear. It was also shown that Langmuir monolayers of lysozyme formed on the liquid surface also have a thickness comparable to the size of the octamer formed in the crystallization solution with the addition of potassium chloride (KCl) precipitant. The octamer is not destroyed when the crystallization solution is applied to the surface of the liquid, which confirms the calculation of the stability of the lysozyme octamer carried out using the molecular dynamics method [12]. It was also shown that the precipitant ions K+ and Cl – form thin layers near the protein film, with the Cl – ion layer being closer to the film than the potassium ion layer [13]. Such a difference in the behavior of the cations and anions of the precipitant indicates that the lysozyme molecules in the film have a positive surface charge.

In this work, with the aim to reveal the orientation of octamers in the lysozyme layer, a new approach to process X-ray reflectometry data is taken. The approach is based on the use of the atomic structure information of lysozyme octamers. The approach is tested for lysozyme films formed on silicon substrates from solutions with the addition of NaCl precipitant under the crystallization conditions of the tetragonal phase presented in [11]. A study is also made to carry out of the Langmuir layers of lysozyme formed from crystallization solutions with the addition of copper (II) chloride and nickel (II) chloride (CuCl2) (NiCl2) precipitants on the surface of a liquid (water) subphase with the aim to observe the specific features of the interaction of salt ions with protein layers.

2. Materials and methods

2.1. Preparation of lysozyme solutions

Chicken egg lysozyme (HEWL) (CAS #12650-88-3, Sigma–Aldrich) was used to form protein films. Copper (II) chloride (CAS no. 7447-39-4, Acros Organics), Nickel (II) chloride (CAS no. 7791-20-0, Alta Aesar) and sodium acetate (CAS #6131-90-4, Helicon) were used to prepare the protein solutions. Ultrapure water, obtained with the Simplicity 185 purification system (Millipore, water resistance 18MΩ*cm), was used to prepare buffers. All solutions used in this work were obtained from two initial solutions: 80 mg/ml lysozyme in sodium acetate buffer (0.2 M CH₃COONa/CH₃COOH, pH 4.5; centrifuged for 10 min at 10,000 revolutions/min) and 0.4 M CuCl₂ and 0.4 M NiCl₂ in sodium acetate buffer (filtered through a membrane filter (Millex) with a 0.22-μm pore diameter). The mixed lysozyme–CuCl₂ and lysozyme–NiCl₂ solutions parameters (protein and salt concentrations, pH) corresponded to the growth conditions used for generating tetragonal lysozyme crystals. This solution was used to prepare a lysozyme monolayer with CuCl₂ and NiCl₂ on the subphase surface. Details of the preparation of lysozyme films on solid substrates with NaCl precipitant are given in [11].

2.2. GI-XSW measurements

The GI-XSW measurements were performed at the “Langmuir” beamline at the Kurchatov source of synchrotron radiation (NRC Kurchatov Institute, Russia) using Langmuir trough Nima 601A.
We used a monochromatic X-ray with $E=13$ keV to excite S $K\alpha$, Cl $K\alpha$, and K $K\alpha$ X-ray fluorescence. Reflectivity and fluorescence data were simultaneously collected for angles of incidence of the liquid surface, ranging from 0 to 0.13°. The X-ray fluorescence spectra were collected using a VORTEX SDD detector, and the specularly reflected data were recorded using a scintillation detector. The total data-collection time for each point was 300 s. To reduce the background fluorescence signal from air scattering, the Langmuir trough containing the sample was placed in a cell with a helium atmosphere. This cell comprised an airtight plexiglass box with windows made of thin X-ray-transparent foil and with channels for the supply and input of gases. The air in the cell was replaced with helium by creating excess helium pressure supplied to the cell. The air was discharged through a hydraulic lock. To allow access to the trough, the cell was equipped with a removable hood. After the deposition of the protein solution on the surface of the water subphase in the trough, the helium cell was sealed, and helium was introduced. The degree of air displacement was estimated by the decrease in the intensity of the Ar $K\alpha$ line in the fluorescence spectrum, which was excited when the X-ray beam passed through the air. The synchrotron beam was oriented parallel to the surface of the trough using X-ray optical elements. The barrier of the Langmuir trough was used to press the monolayer down to a predetermined value. The X-ray experiment was then begun, and the surface pressure was held constant during the measurements. Active vibration protection was used to reduce the vibration of the Langmuir trough. The measurements were obtained at room temperature.

2.3 Experimental X-ray reflectometry and GI-XSW Data analysis

A model-dependent approach for XRR and GI-XSW experimental data processing was used. The electron density profile model of studied films was chosen based on simulated electron density distribution of lysozyme protein molecules (monomers, octamers) [PDB ID 6QWY (lysozyme with NaCl), 6QWW (lysozyme with CuCl$_2$) and 6QWX (lysozyme with NiCl$_2$)]. The electron density profiles of studied films were obtained by searching for the best fit between XRR experimental data and model dependent XRR curves calculated using Parratt formalism [14]. Based on the received electron density profiles XSW field distributions in studied films were calculated and atomic species concentration distributions were obtained by fitting the experimental and calculated X-ray fluorescence yield curves. XRR and GI-XSW curves fitting was performed using Levenberg-Marquardt algorithm. [15].

3. Results and discussion

3.1. Structure of lysozyme – NaCl films on silicon substrates.

As a result of modeling it was found that electron density distribution in lysozyme octamer varies depending on the chosen molecule orientation (Fig. 1). Based on various calculated octamer electron density distributions an electron density profile model of studied protein films was chosen. The use of a new model made it possible to reduce the difference between experimental and calculated XRR data compared to the previous study [11]. It is also made it possible to obtain more detail information about films structure, namely, to clarify the molecules orientation by comparing theoretical octamer electron density distribution (PDB ID 6QWY) and received electron density profiles of studied films (Fig. 2).
Figure 1. Electron density distribution in lysozyme octamer (PDB ID 6QWY) along the fourfold symmetry (a) and twofold symmetry axis

Figure 2. Experimental and theoretical XRR curves (a) and electron density profiles obtained after fitting (b) for lysozyme film deposited onto the silicon substrate from crystallization solution with NaCl (experimental XRR curve is given in [11]).

Thus, the data obtained in accordance with the proposed approach made it possible to refine the structure of lysozyme protein films on solid substrates in comparison with the approach used earlier (without taking into account data on the atomic structure of lysozyme molecules). Using the received more accurate electron density profile the GI-XSW data obtained in the previous work [11] was processed (fig. 3).
a) Figure 3. (a) – GI-XSW experimental and calculated data, (b) – atomic species concentration distributions obtained after fitting for the lysozyme film formed from crystallization solution with the addition of NaCl and deposited onto the silicon substrate (experimental GI-XSW data are given in [11]).

Thus, it has been possible to show that the octamers in the lysozyme film have a preferential orientation and are arranged in such a way that the fourfold axis symmetry is perpendicular to surface of the substrate (Fig. 4). Moreover, the data obtained by the GI-XSW method reveal the mutual arrangement of the precipitant ions in the lysozyme layer structure and differ only slightly from those obtained previously.

Figure 4. The model of octamers oriented in the protein film on silicon substrate according the analysis of X-ray reflectivity data. The octamers orientate with their fourfold axis symmetry perpendicular to the substrate surface.

3.2. Calculation of octamer’s surface potential

It was shown in [13] that when Langmuir layers are formed from a crystallization solution of lysozyme with a potassium chloride precipitant, potassium and chloride ions form thin layers near the lysozyme layer, with Cl\(^-\) anions being more closely adjacent to the protein than K\(^+\) cations. This, in turn, shows that lysozyme molecules have a positive charge. To determine the predominant charge in the octamer, the electrostatic potential of the lysozyme octamer was calculated using the APBS – PDB2PQR software package [16], which calculates the ion charge and their radius based on the atomic structure of the protein contained in the PDB file. The calculation was carried out at a pH of 7, which approximately corresponds to the pH of the subphase. The potential distribution was visualized.
using the PyMol (PyMOL Molecular Graphics System, Version 1.8, Schrödinger LLC) program. It was calculated that the lysozyme octamer predominantly has a positive surface charge, which is consistent with the fact that negatively charged chloride ions are more strongly attracted to the protein layer than positively charged potassium ions. The predominantly positive charge of the lysozyme octamer is also explained by the fact that the protein is in a medium with a pH whose value is less than the value of the isoelectric point of lysozyme (IT ≈ 11.00). The images show the distribution of the potential in the upper and lower parts of the octamer. It can be seen that in the lower part there are polar positive regions (Fig. 5a (c), blue color), and in the upper part of the octamer the negatively charged (Fig. 5a (b), red color) and uncharged hydrophobic parts (white color) are concentrated.

**Figure 5.** (a) Distribution of the surface electrostatic potential of the lysozyme octamer: a) side view, b) top view and c) bottom view. Blue color corresponds to positively charged areas, red color to negatively charged areas, white color to uncharged areas; (b) Model for the orientation of the lysozyme octamer on the surface of the liquid in the Langmuir monolayer, taking into account the distribution of surface potential.

Based on this, it can be assumed that the octamer is oriented to the liquid in such a way that their fourfold axis symmetry perpendicular to the surface of the liquid (Fig. 5b), which also agrees with the results of processing the data presented earlier (Fig. 4). The attraction of the octamers in the Langmuir layer may be due to the presence of hydrophobic interactions between the octamers that arise between the nonpolar parts of the molecules, as a result of which the system reaches its maximum thermodynamic stability by minimizing the number of ordered water molecules necessary to surround the hydrophobic parts of the dissolved molecules. This assumption is consistent with the conclusions made based on molecular modeling calculations that the final thermodynamic stability of a protein crystal is ensured by hydrophobic short-range contacts [17].

3.3. Langmuir lysozyme films with CuCl2 and NiCl2 precipitants

Using the GI-XSW method, the structure of the Langmuir lysozyme layers formed from crystallization solutions with the addition of precipitants CuCl2 (Fig. 6) and NiCl2 (Fig. 7) on the liquid surface was studied. To determine the location of lysozyme molecules, a signal was recorded from sulfur, which is contained in a number of amino acid residues of lysozyme (Cys-6, Met-12, Cys-30, Cys-64, Cys-76, Cys-80, Cys-94, Met-105, Cys-115, and Cys-127).

In case of using crystallization solution with the addition of CuCl2 precipitant to form the films, the GI-XSW data shows that the film thickness is about 110 Å. This thickness is comparable with the processing data obtained by processing models of octamers oriented with their fourfold axis symmetry perpendicular to the liquid surface (Fig. 2b).
The angular dependences of the X-ray fluorescence yield of sulfur (a), chlorine and copper (b) of the Langmuir lysozyme layer formed from a crystallization solution with CuCl₂ precipitant.

![Graph](image1)

**Figure 6.** The angular dependences of the X-ray fluorescence yield of sulfur (a), chlorine and copper (b) of the Langmuir lysozyme layer formed from a crystallization solution with CuCl₂ precipitant.

The angular dependences of the X-ray fluorescence yield of sulfur (a), chlorine and nickel (b) of the Langmuir lysozyme layer formed from a crystallization solution with NiCl₂ precipitant.

![Graph](image2)

**Figure 7.** The angular dependences of the X-ray fluorescence yield of sulfur (a), chlorine and nickel (b) of the Langmuir lysozyme layer formed from a crystallization solution with NiCl₂ precipitant.

Determination of the positions of precipitant ions made it possible to detect the presence of a specific interaction between copper ions and lysozyme molecules, which manifests itself in overlapping distribution profiles of copper and sulfur elements. At the same time, chlorine ions are displaced closer to the film than copper ions, suggesting that lysozyme molecules have a predominantly positive charge, as previously calculated (Fig. 5). Chloride and copper ions penetrate the protein layer (Fig. 8), from which it can be concluded that in this case, the interaction of the precipitant ions containing a divalent metal is more pronounced than in the case of monovalent chlorides of potassium and sodium metals. The chlorides of divalent metals are assumed to have a significant effect on the structure of the protein film.

Similar results are also observed in the case of studying the Langmuir layers of lysozyme formed from crystallization solutions with the addition of NiCl₂ precipitant (Fig. 7b and 8b). The film thickness in this case is also 110 Å approximately. This thickness is also comparable with the processing data obtained by processing models of octamers oriented with their fourfold axis symmetry.
perpendicular to the surface of the water subphase. Chloride and nickel ions in the case of NiCl$_2$ precipitant behave in the same way as copper and chlorine ions of CuCl$_2$ precipitant.

**Figure 8.** Distribution profiles of sulfur (S, blue curve), chlorine (Cl, red curve), copper (Cu) and nickel (Ni) (green curves, corresponding (a) and (b)) modeled on the basis of the octamer structure (PDB ID 6QWW and 6QWX) over the thickness $z$ of the Langmuir layer of lysozyme, formed from a solution with the addition of CuCl$_2$ and NiCl$_2$ precipitants, on the surface of a water subphase when the octamer is oriented with their fourfold axis symmetry perpendicular to the surface of the water subphase.

The obtained results show that the presence of a divalent metal in the precipitant has a significant effect on the structure of the film, and, most likely, such ions will also affect the behavior of oligomers in solution and the self-organization of lysozyme molecules during crystallization, which was confirmed by the study of lysozyme solutions by small-angle X-ray scattering [18].

### 4. Conclusion

A method for processing X-ray reflectivity and grazing-incidence X-ray standing waves data for protein films has been developed and applied. This approach consists of choosing the primary layered model of the studied film based on the electron density modelling of the structure of lysozyme oligomers isolated from the crystal lattice using structural information about their structure. This method was used to process data obtained by studying the structure of lysozyme films on solid substrates formed using the modified Langmuir-Schaeffer method developed previously. The essence of the method is to use crystallization solutions of lysozyme protein with the addition of a precipitant, the parameters of which (protein and precipitant concentrations, buffer type, etc.) correspond to the crystallization conditions of the selected protein. This method made it possible for the first time to obtain multilayer thin-film systems consisting of lysozyme molecules and layers of precipitant ions on a solid substrate and on the surface of an aqueous subphase in a Langmuir through from polydisperse crystallization solutions (containing pre-crystallization clusters — protein oligomers). It was shown that the octamers involved in the formation of the protein film have a preferential orientation and are directed so that their fourfold axis symmetry is perpendicular to the substrate surface. Thus, a method of processing experimental data based on the use of structural information of protein oligomers has revealed the predominant orientation of protein molecules in the layer. Also, using the method of grazing-incidence X-ray standing waves, the structure of the Langmuir layers of lysozyme formed from crystallization solutions with the addition of copper and nickel chlorides precipitants was studied. It was shown that the lysozyme layers have a thickness comparable to the size of the octamer, and precipitating ions have a significant effect on the structure of the obtained protein films.
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