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Coherent anti-stokes Raman spectroscopy of nanodiamond–lysozyme interactions in water

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Abstract

The paper presents the results of studying the interactions of oxidized nanodiamonds with lysozyme in water. It was found that lysozyme is actively adsorbed on the surface of nanodiamonds in two layers. For the first time, using the CARS method, it was found that the conformation of lysozyme molecules in the first adsorption layer changes, and the conformation of molecules adsorbed in the second layer almost coincides with the conformation of free lysozyme molecules. The obtained results and the analysis of the literature data concerning the activity of lysozyme allowed us to conclude that lysozyme in the first adsorption layer mainly loses its enzymatic activity, and in the second—retains it.

Keywords: coherent anti-stokes Raman spectroscopy, nanodiamonds, lysozyme

(Some figures may appear in colour only in the online journal)

1. Introduction

Currently, new nanocomplexes for simultaneous diagnostics and treatment of diseased cells are being actively developed [1–3]. Most often, such theranostic agents are photoluminescent nanoparticles, with drugs or bioactive molecules attached to their surface [2, 3]. Due to the intense and stable photoluminescence [4, 5], high adsorption properties [6, 7] and nontoxicity [7], carbon nanoparticles, including detonation nanodiamonds (DND) [8], are the most promising for use in biomedicine. In addition to the initial adsorption abilities of DND, the appropriate functionalization of the surface provides them high selective adsorption activity with respect to metal ions [9], drugs [10, 11], proteins and DNA molecules [12, 13], etc. Moreover, in many publications it was noted that many of these substances interact with the DND surface by physical adsorption [6, 10, 12].

The creation of theranostic nanocomplexes—attachment of bioactive molecules to solid surfaces—is one of the main problems of modern biology and medicine. According to the literature data, as a result of such immobilization, the lifetime of enzymes increases by orders of magnitude, their stability in the medium in relation to changes of temperature, pH, etc, increases [12, 14]. If biomolecules or drugs are immobilized on the nanoparticle in a certain way, their functional activity is almost completely preserved [15]. These results have become a incentive for the development of complexes consisting of nanodiamonds with lysozyme (antibacterial enzyme that catalyzes the destruction of the cell walls of gram-positive bacteria) immobilized on their surface [16, 17].

The interactions of lysozyme with the surface of nanodiamonds are actively studied by many groups [16–22]. As it is known, for applications in biomedicine, DNDs

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with carboxylated surface are usually used as the most biocompatible. It was found that for such DNDs the main mechanism of adsorption of protein molecules is the electrostatic interaction between positively charged amino groups $–NH_3^+$ on the protein surface and negatively charged carboxyl surface groups $–COO^-$ of nanodiamonds [18, 21]. Thus, lysozyme can interact with the surface groups of DND by physical adsorption [18, 21], as a result of the formation of hydrogen bonds between DND and lysozyme [19], or by hydrophobic attraction [22].

When lysozyme is immobilized on the surface of nanoparticles, the problem of preserving the activity of the enzyme as a result of adsorption arises. Obviously, it depends on the way the lysozyme molecules are 'arranged' on the DND surface. Most authors consider that lysozyme is adsorbed on the DND in a single layer [18, 19, 21]. However, in the publication [22] it was shown for the system 'sensor-SiO_2 layer-nanodiamond layer-lysozyme layer', that on the surface of DND with oxygen-containing groups, lysozyme can be adsorbed in two layers, and on the surface of DND with hydrogen—in three layers. Moreover, in the first layer, the lysozyme molecule is located 'along' the surface of the nanoparticle (lysozyme molecule has a shape similar to cylindrical). The authors of publications [18, 19, 21, 23] showed that lysozyme in the adsorbed state on the surface can lose its activity. According to the authors [18], the enzymatic activity of lysozyme significantly decreases when the protein is adsorbed on nanodiamonds with sizes smaller than 50 nm. This is evidenced by shifts of the positions of the amide bands I and II of lysozyme in the IR absorption spectra. Such shifts are caused by changes of the conformation of protein.

When studying the interactions of nanodiamonds and components of biological media (proteins), the methods of vibrational spectroscopy–Raman spectroscopy and IR absorption spectroscopy—are widely used. These methods complement each other and allow to obtain information about the structural changes of the studied molecules. However, the use of the method of spontaneous Raman spectroscopy is significantly limited by two factors—the weakness of the signal and the need to separate useful signal against the background of fluorescence of the studied molecules. The latter factor often plays a critical role in case of the excitation of Raman signal by visible radiation.

In order to expand the capabilities of vibrational spectroscopy, the method of coherent anti-stokes Raman scattering (CARS) is used. This method is based on the nonlinear interaction of two laser beams with frequency detuning coinciding with the frequency of the studied molecular vibration. CARS is a resonant case of four-wave nonlinear optical process in which pump photons ω_p, Stokes wave photons ω_s, and probing radiation ω_{pr} interact in such a way that signal is generated in the anti-stokes part of the spectrum $\omega_{as} = (\omega_p - \omega_s) + \omega_{pr}$, when the frequency difference $\omega_p - \omega_s$ falls into the vibrational resonance of molecules of the substance Ω_R. As a result, when the synchronism condition is satisfied, the anti-Stokes Raman scattering signal at the selected frequency increases many times in comparison with the case of spontaneous Raman scattering [24]. Moreover, since the method involves the use of the anti-stokes region of the scattering spectrum, the problem of autofluorescence is eliminated, and the narrow directivity of the radiation and the small overlap region of the pump waves in the sample allow one to obtain a signal from small volumes. This nonlinear process was firstly observed in [25] in 1965, and then became actively used in various studies [26, 27].

Method of CARS is particularly actively used in the study of biomolecules. Thus, in [28], CARS was used for studying the amide-I band of solutions of various proteins (lysozyme, albumin, etc) in deuterated water. It was shown that the use of polarization-sensitive measurements in the implementation of CARS makes it possible to distinguish in the spectrum of the amide-I band the components corresponding to different secondary structures of the protein. In [29], the possibilities of CARS spectroscopy for protein identification and quantitative analysis of amino acids are shown. The creation of the CARS microscope [30] made it possible to implement a new method of optical imaging at the cellular level. Now this method is widely used in cell imaging [31], as well as for the visualization of nanoparticles in cells [32].

This article presents the results of the study of the interaction of lysozyme with the surface of oxidized nanodiamonds in water by method of CARS. It is found that stable complexes are formed as a result of the adsorption of lysozyme on the surface of nanodiamonds. The conformational state of the protein during adsorption to the surface of nanoparticles is analyzed.

2. Experiment

2.1. Objects of research

In this study oxidized nanodiamonds produced by detonation method of synthesis (Adámas Nanotechnologies (Raleigh, USA)) with carboxyl and hydroxyl groups on the surface were used. Chicken egg protein lysozyme (Ultra pure grade) was purchased from Amresco (Cat#: 0663; CAS#: 12650-88-3; activity > 20000 μ mg^{-1}).

An aqueous suspension of DND with concentration of 1 mg ml^{-1} was prepared. The sizes of nanoparticles in the initial suspension were measured by dynamic light scattering. They were found to be 31.0 ± 0.2 nm. The zeta potential of the prepared DND suspension was −38.0 ± 0.5 mV.

Aqueous solutions of lysozyme with concentration of the enzyme varied in the range from 10^{-3} to 2 mg ml^{-1} were prepared. According to the literature data [33], in aqueous solutions lysozyme has an almost ellipsoidal shape with sizes of 3 × 3 × 4.5 nm.

2.2. CARS experimental setup

The scheme of experimental setup for registration of CARS signals is shown in figure 1. Degenerate CARS ($\omega_p = \omega_{pr}$) was implemented: the second harmonic (532 nm) of pulsed Nd:YAG laser (model LQ629-100, Solar Laser Systems, Belarus) with a pulse duration of 10 ns and a pulse repetition rate of 100 Hz was used as pump and probe sources. The third harmonic (355 nm) of the same Nd:YAG laser was used to pump optical parametric oscillator (OPO) LP603 (Solar Laser

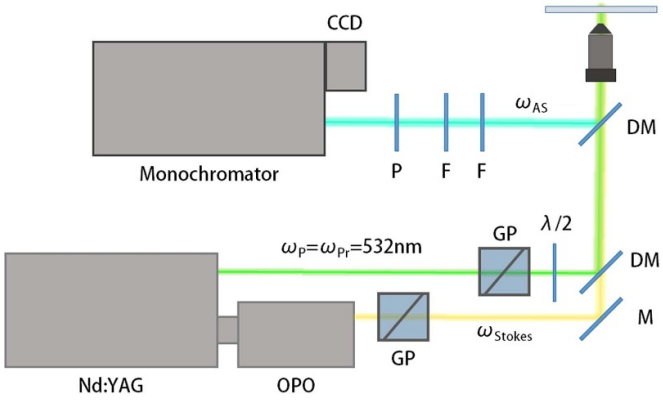

Figure 1. Experimental setup. GP—Glan prism, M—mirror, DM—dichroic mirror, F—filter, P—polarizer.

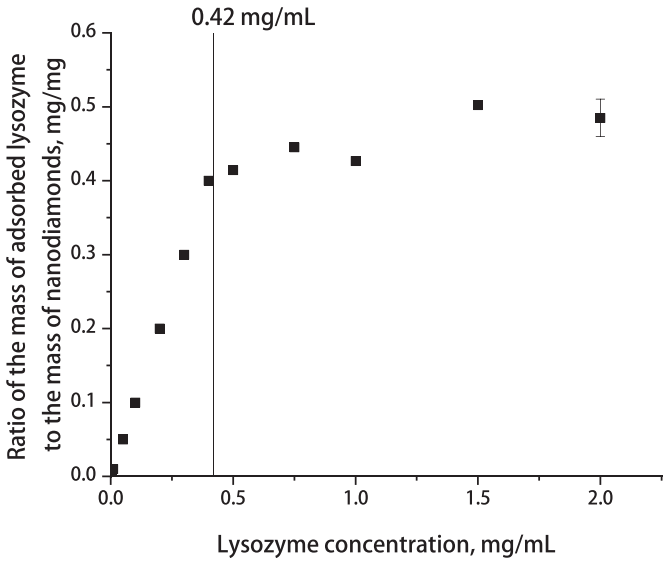

Figure 2. Isotherm of lysozyme adsorption on the DND surface in aqueous suspension at pH = 4.7.

Systems, Belarus), the radiation of which was used as Stokes wave ω_s in the CARS.

In the experiment on the interaction of ND with lysozyme, the wavelength of the Stokes component varied in the range from 575 to 585 nm. The polarization of the Stokes component was linear horizontal and kept constant, and the polarization of the pump radiation was changed by rotating the WPMH05M half-wave plate (Thorlabs). The radiation power of the pump and the Stokes component were adjusted by turning the Glan–Taylor GT10A prisms (Thorlabs). The beams of ω_p, ω_{pr}, ω_s were focused in the sample volume using an achromatic objective with an aperture of 0.4.

CARS signal was obtained in the epi-detection scheme (ECARS). A set of filters (FES0550, NF533-17) was used to separate the signal only in the anti-stokes region. A polarizer was placed in front of the detection system for choosing the specific polarization of the incident radiation. The angle between the polarizations of the ω_p and ω_s beams was 71.21°, and the polarizer transmitted radiation with linear polarization at an angle of 60.11°. As it was shown earlier by the authors [28, 34], it provides the maximum contrast of the spectrum.

The system of registration of CARS spectrum consisted of the monochromator with focal length of 500 mm, assembled according to the Czerny–Turner scheme, equipped with 600 lines mm^{-1} grating and CCD camera (Horiba–Jobin Yvon, Synapse BIUV). The practical spectral resolution of the experimental setup was 4 cm^{-1}.

3. Results and discussion

3.1. Study of lysozyme adsorption on the DND surface

Adsorption of lysozyme on the DND surface in aqueous suspensions at pH = 4.7 was studied. Zeta-potentials of the oxidized DND and lysozyme in water were −38 mV and +24 mV, respectively. Basing on the obtained values of the zeta-potentials, it can be assumed that lysozyme will actively interact with the surface of the studied DNDs by physical adsorption.

For realization of lysozyme adsorption on the DND surface, the initial suspensions of DND and lysozyme were mixed so that DND concentration in the resulting mixtures was constant (1 mg ml^{-1}), and the concentration of lysozyme varied in the range from 10^{-3} to 2 mg ml^{-1}. The mixtures were gently stirred in the XIN BAO XH-C shaker for 2 h. The resulting DND sediment with the adsorbed lysozyme was then separated from the supernatant containing the non-adsorbed lysozyme in the centrifuge. The concentration of lysozyme before its adsorption and in the supernatant after adsorption was measured using spectra of the optical density of the samples.

Basing on the obtained results, adsorption isotherm was constructed—the dependence of the ratio of the mass of the adsorbed lysozyme to the mass of nanodiamonds on the concentration of lysozyme (figure 2). The course of the adsorption isotherm indicates that at any lysozyme concentration less than 0.42 mg ml^{-1}, all lysozyme in the suspension is adsorbed on the surface of the nanodiamonds. At lysozyme concentrations greater than 0.42 mg ml^{-1}, the amount of the adsorbed enzyme decreases significantly: the adsorption saturation occurs (figure 2).

Thus, from the obtained isotherm of lysozyme adsorption to the DND surface, it follows that at certain concentrations of lysozyme, the enzyme adsorption on DND surface becomes saturated.

Using a simple model, it is possible to estimate how many layers of lysozyme are adsorbed on the surface of the studied DND. Let us consider DND as spherical particles with a density of 3.18 g cm^{-3} [35], and lysozyme as cylindrical particles with dimensions of 3 × 3 × 4.5 nm [33]. Note that the lysozyme can be located on the DND surface in two ways: (a) the long side along the DND surface (side-on (∥)); (b) the long side perpendicular to the surface of the nanoparticles (end-on (⊥)). Using all the available geometric data of the studied nanoparticles and lysozyme, the DND surface areas and lysozyme concentrations required to cover the DND

in one, two, and three layers for all these variants were calculated. The results of the calculations showed that the first method—(∥)—requires 0.1 mg ml^{-1} of lysozyme to cover the DND surface in one layer, and the second method—(⊥)—0.16 mg ml^{-1} of lysozyme. The adsorption of lysozyme on the DND surface in two layers can occur as follows: (∥–∥), (∥–⊥), (⊥–∥), (⊥–⊥). These two-layer lysozyme adsorption options will require 0.33 mg ml^{-1}, 0.44 mg ml^{-1}, 0.46 mg ml^{-1}, and 0.61 mg ml^{-1}, respectively. To cover the DND surface in more layers, lysozyme concentration of more than 0.72 mg ml^{-1} is required.

From the comparison of the lysozyme concentration at which the adsorption on the DND surface becomes saturated (∼0.42 mg ml^{-1}, figure 2) and the calculated values of the lysozyme concentrations, it follows that this concentration corresponds only to the adsorption of lysozyme in two layers in the configuration (∥–⊥). Significantly higher concentrations of lysozyme are required to fill the third layer. Thus, in the first layer of adsorption, the most likely position of the lysozyme is the side-on position, and in the second layer, the position is perpendicular to the DND surface—end-on.

3.2. Investigation of the conformation of lysozyme adsorbed on the DND surface using CARS method

When lysozyme is adsorbed on the surface of nanoparticles, the problem of preserving the activity of the enzyme arises. The activity of lysozyme depends on the conformation of the protein, which, in turn, depends on the way the lysozyme molecules are 'arranged' on the DND surface. As it is known, conformational changes of the protein manifest themselves in changes of the position of the amide lines of proteins. In order to determine the conformation of the adsorbed enzyme in the DND + lysozyme complex, a comparative analysis of the amide lines I and II of free and adsorbed lysozyme was performed using CARS method.

Aqueous solutions of lysozyme and suspensions of DND + lysozyme complexes were applied to the surface of the slide and dried to a paste-like consistency. This was necessary in order to weaken the intensity of the band of deformation vibrations of the OH groups of water in the region of ∼1630 cm^{-1}, which partially overlaps with the line of amides I (with a maximum in the region of 1640 cm^{-1}), and secondly, to reduce the non-resonant signal from water as much as possible in the recorded epi-CARS spectra.

Figure 3 shows the amide band I (figure 3(a)) and II (figure 3(b)) of lysozyme obtained by the CARS method in the free state and adsorbed on the DND surface. The lysozyme concentration in the samples was 0.05 mg ml^{-1}, and the DND concentration was 1 mg ml^{-1}. Each of the presented bands is an averaging over 15 spectra, sequentially recorded during the experiment at three spatially different points of the sample. The processing of each spectrum consisted of normalizing the spectral sensitivity of the CCD camera and smoothing the bands by five points by the Savitsky–Galey method.

The analysis of the amide lines I (figure 3(a)) and II (figure 3(b)) of lysozyme obtained by CARS method in the

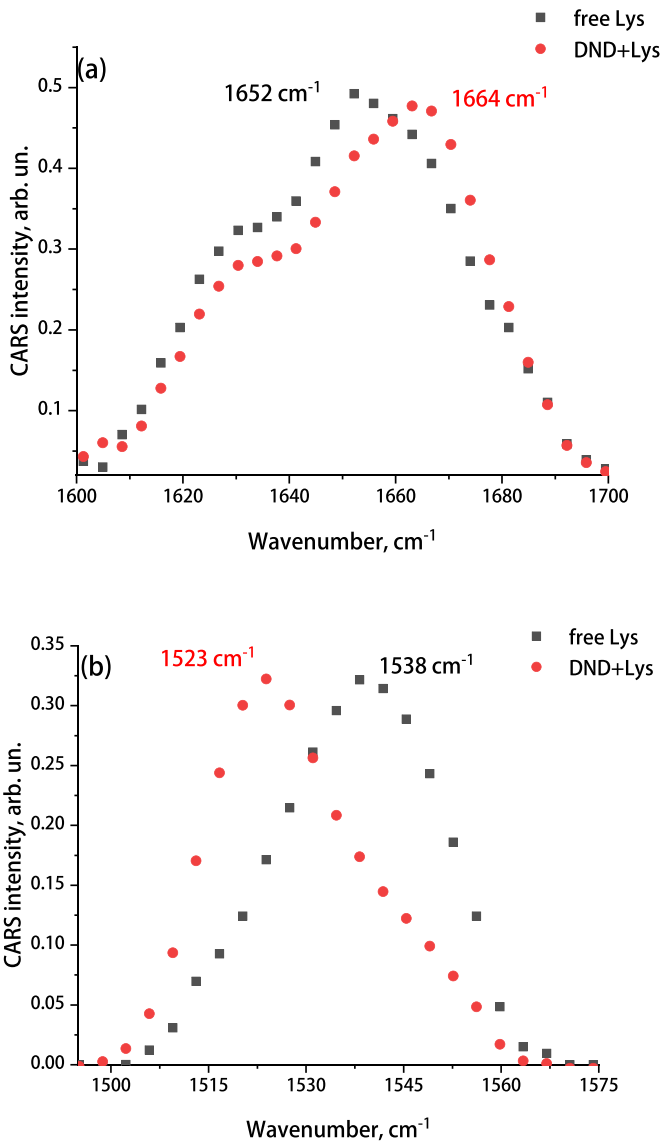

Figure 3. Amide lines I (a) and II (b) of lysozyme obtained by CARS method in the free state and adsorbed on the DND surface. The concentration of lysozyme in the samples is 0.05 mg ml^{-1}.

free state and adsorbed on the DND surface shows that at the concentration of lysozyme in the samples 0.05 mg ml^{-1}, the amide lines I and II of lysozyme adsorbed on the DND surface shift relative to those of lysozyme in the free state. This means that the state of the protein changes as a result of its interaction with the surface.

Figure 4 shows the dependence of the position of the maximum of the amide lines of lysozyme on its concentration, varying from 10^{-3} mg ml^{-1} to 2 mg ml^{-1} at fixed DND concentration 1 mg ml^{-1}. As it follows from the obtained data, with an increase of the amount of lysozyme from 10^{-3} mg ml^{-1} to 0.1 mg l^{-1} in the suspension, the amide bands of the protein adsorbed on the DND surface are shifted relative to the bands of free lysozyme in opposite directions: the amide band I towards high wavenumbers by 12 cm^{-1}, the amide band II towards low wavenumbers by 15 cm^{-1}. With a further increase of the amount of lysozyme adsorbed on the

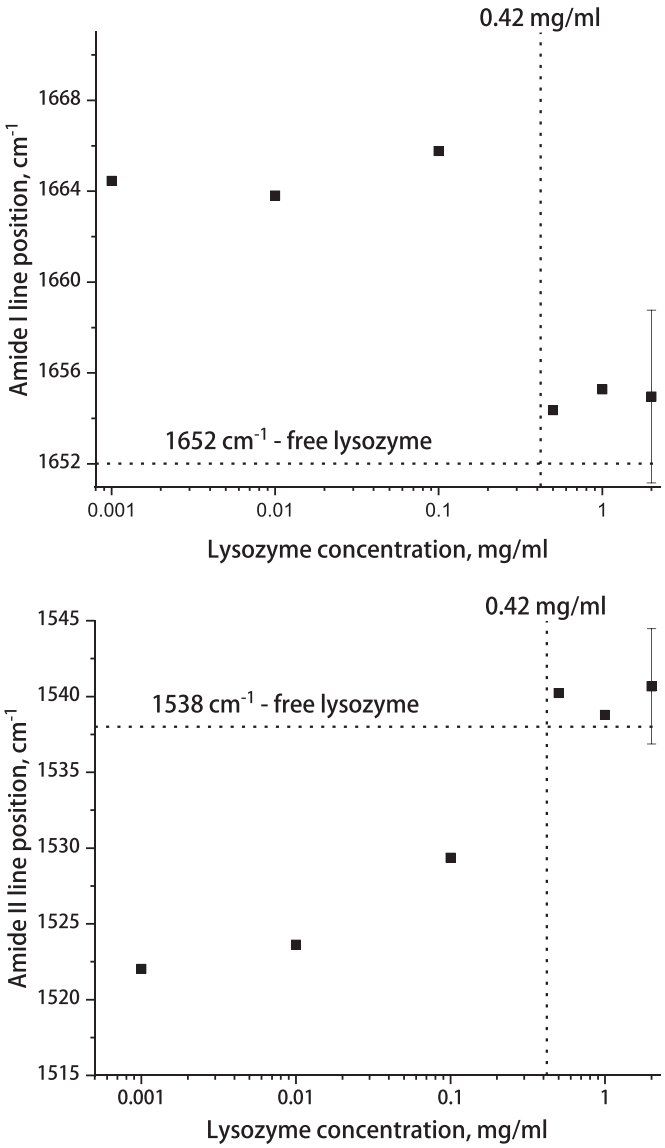

Figure 4. Dependence of the position of amide bands I and II of lysozyme adsorbed on the DND surface on the protein concentration. DND concentration is 1 mg ml^{-1}.

DND surface (according to figure 2, active protein adsorption on the DND continues up to concentration 0.42 mg ml^{-1}), the position of the amide I and amide II bands approaches the position of these bands for free lysozyme.

On the basis of the results of CARS spectroscopy and the estimations of the configuration of the DND + lysozyme complexes, it can be concluded that in the first layer of lysozyme adsorbed on the DND surface (up to protein concentration 0.1 mg ml^{-1} according to calculations), the conformation of lysozyme changes. Highly likely, it is adsorbed on the surface of nanoparticle by the side-on (∥) way, i.e. the protein is unfolded. With an increase of the lysozyme concentration from 0.1 mg ml^{-1}, protein is adsorbed into the second layer on the surface of ND. In this case, the conformation of protein does not change in comparison with the free lysozyme, the protein is adsorbed on the surface by the end-on way (⊥).

Thus, the results of study of interactions of lysozyme and DND in water obtained by CARS spectroscopy confirm the two-layer adsorption of lysozyme on the DND surface [18, 20]. When the amount of lysozyme is small, protein forms a monolayer around the nanodiamond particles, change of the protein conformation occurs, which is manifested in the CARS spectra. After increase of protein concentration, when the second layer of lysozyme begins to form on the DND surface due to adsorption, conformational changes are minimal. Exactly the invariability of the lysozyme conformation in the second adsorption layer ensures the preservation of its enzymatic activity in the DNA + lysozyme complex. The obtained results are qualitatively consistent with the results of publications [18, 20].

4. Conclusions

In this study, as a result of studying the interactions of oxidized nanodiamonds with lysozyme in water, it was demonstrated that lysozyme is actively adsorbed on oxygen-containing surface groups of the DND. It was found that protein is adsorbed on DND surface in two layers. Numerical estimations of the number of lysozyme molecules required to cover the DND in two layers are in good agreement with the obtained adsorption isotherm and results of CARS spectroscopy of DND + lysozyme complexes only if the lysozyme molecules in the first adsorption layer are located by the side-on (∥) way, i.e. their long side is parallel to the surface, and in the second layer—perpendicular to the surface (by the end-on (⊥) way).

For the first time, using CARS method, it was found that conformation of lysozyme molecules in the first adsorption layer is changed, and the conformation of molecules adsorbed in the second layer almost coincides with the conformation of free lysozyme molecules. The obtained results and analysis of the literature data on the activity of lysozyme allowed us to conclude that lysozyme in the first adsorption layer mainly loses its enzymatic activity, and in the second—retains it.

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