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Features of the Local Immobilization of NADPH-Cytochrome P450 Reductase and Cytochrome b_5 on a Solid Surface

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Abstract—Using the methods of microcontact printing and atomic force microscopy, we studied a possibility of specific "protein-protein" interactions in the processes of local immobilization of cytochrome b_5 and NADPH-cytochrome P450 reductase on the surface of hydrophilic silicone. It is shown that the formation of protein complexes is possible only at a certain orientation of the molecules defined by the sequence of adsorption of biopolymers.

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The problem of immobilization of oxidoreductases in the native conformation on a solid surface is the key to the development of the biosensors operating on the principle of reversible electron transfer. However, the mechanisms of these processes, including the features of interaction of various components of such enzyme systems are still insufficiently studied [1, 2]. A promising approach to obtaining an information about the biological objects in the form of images on a solid surface at the study of the samples both in air and in fluids under the conditions close to physiological, is the application of atomic force microscopy (AFM) [3], which is widely used in biology and medicine for the analysis of crystals of amino acids, proteins, cell membranes, bacteria, and viruses [3–5]. For proper evaluation by the AFM method of the size of proteins, their orientation, and possible changes in the structure at the interaction of molecules with partners, we used the reference samples with micron-sized sections of the hydrophilic silicone surface, created using microcontact printing (MCP) [6, 7].

The purpose of this work was to explore the features of interaction of cytochrome b_5 and NADPH-cytochrome P450 reductase on the solid surface, as well as the possibility of formation on the hydrophilic silicone of the respective protein complexes.

The microstructured films of the chimeric proteins Hmwb5-LmwCPR, Hmwb5-HmwCPR and Lmwb5-LmwCPR (chimeric protein means a protein synthesized artificially by genetic engineering, in which from a single polypeptide chain are formed two functionally independent domains that in a certain degree retain the structure of the initial proteins naturally occurring separately) were studied by AFM (Fig. 1a).

It should be noted that traditionally the thickness of the protein monolayer is determined from the depth of a natural or artificially formed defect in the film structure [8], created by scanning a small area (usually 400 nm) at the strength of the needle impact on the sample no less than 10 nN. It is assumed that the material of the film formed on the spot of the defect is removed completely. The application of the MCP method allows the creation of the microstructured monolayers in which the areas occupied by the film alternate with the areas of the pure silicone with 3 μm step [9]. Therefore, in the latter case, the analysis of cross-section profile is more correct. The table lists the data on the thickness of the monolayers for all the investigated proteins.

We showed that the chimeric proteins in the form of microstructured monolayers on silicone can block

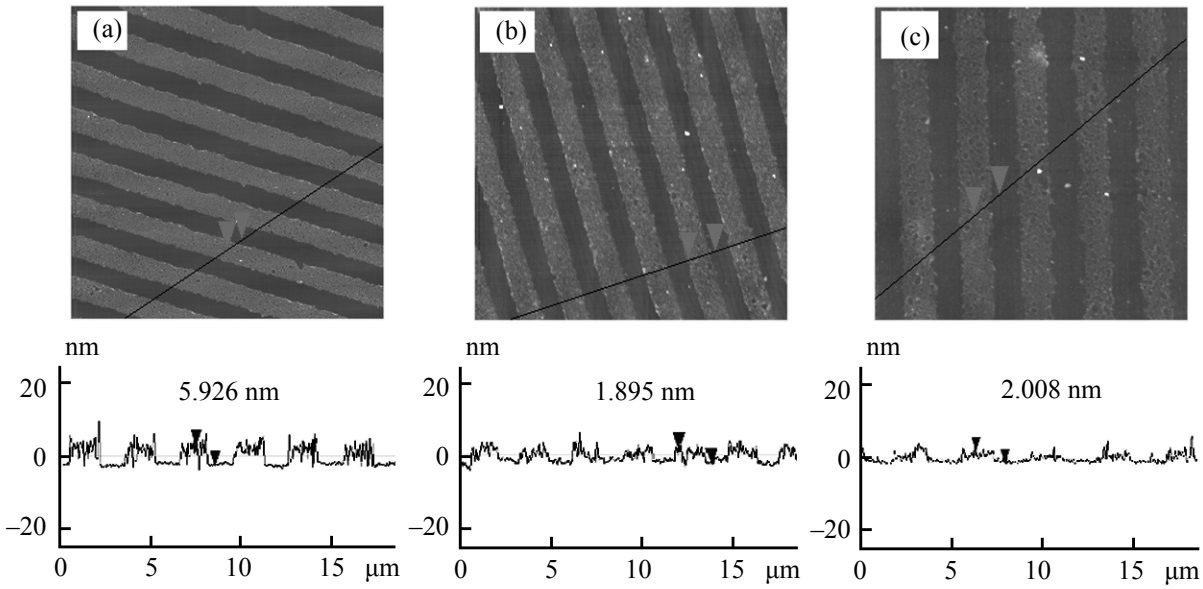

Fig. 1. AFM images and profiles of cross-sectional microstructure of the films of chimeric protein $Hmwb_5$-LmwCPR on silicone: (a) before treatment, (b) after treatment with a solutions of NADPH-cytochrome P450 reductase, and (c) after treatment with a solutions of NADPH-cytochrome P450 reductase and cytochrome b_5.

further adsorption of the individual full-length proteins with the fragments occurred in the structure of the chimeric protein. Thus, at the processing of microstructurized films of a chimeric protein with a solution of individual protein the step height (the difference between the thickness of the monolayers) is proportional to the molar mass (and hence to the size) of the individual protein. For example, of processing the microstructured film $Hmwb_5$-LmwCPR with a solutions of NADPH-cytochrome P450 reductase or of cytochrome b_5, the step height was reduced to ~4 and ~2 nm, respectively (Figs. 1b and 2b). Thus, it can be stated that the adsorption of individual proteins occurs only in the valleys of the micro-relief.

The possibility of specific protein-protein interactions was tested by sequential adsorption of cytochrome b_5 and NADPH-cytochrome P450 reductase on the areas of hydrophilic silicone not blocked by a $Hmwb_5$-LmwCPR monolayer. Thus, after processing the microstructured $Hmwb_5$-LmwCPR film by a solution of NADPH-cytochrome P450 reductase the step height decreased from ~6 to ~2 nm (Fig. 1b), and then, after processing the resulting system with a solution of cytochrome b_5, remained unchanged (Fig. 1c). Thus, the final leveling of the micro-relief, as would be expected at the formation on the surface of the complex NADPH-cytochrome P450 reductase–cytochrome b_5, in this case does not occur.

A different situation was observed when the microstructured Hmwb5-LmwCPR film was processed first by a solution of cytochrome b_5, and then by a solution of NADPH-cytochrome P450 reductase. This gradual decrease in the height of steps at the sequential adsorption of these proteins leads to the eventual leveling of the microrelief (Figs. 2b and 2c). Apparently, the active centers of enzymes thus remain sterically accessible, which determines the possibility of specific intermolecular interactions and formation of the complex cytochrome b_5–NADPH-cytochrome P450 reductase on the surface of hydrophilic silicone.

Thus, by the method of microcontact printing on the surface of silicone film were formed microstructured artificial chimeric proteins consisting of full-cytochrome b_5 and NADPH-cytochrome P450

Thickness of monolayers and its correlation with the molar mass of the corresponding proteins

Protein	Monolayer thickness, nm	Molar mass, g mol^{-1}
$Hmwb_5$–LmwCPR	6.0±0.2	90000
$Hmwb_5$–HmwCPR	6.0±0.2	97000
$Lmwb_5$–LmwCPR	5.0±0.2	81569
Cytochrome b_5	2.0±0.2	16000
NADPH-cytochrome P450 reductase	4.0±0.2	81000

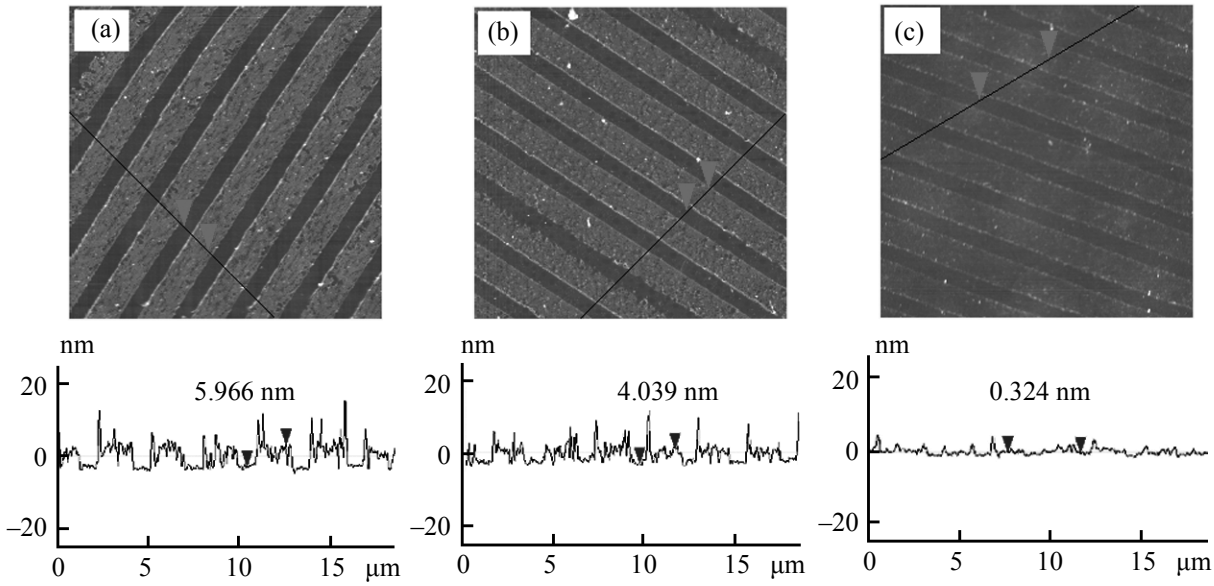

Fig. 2. AFM images and cross-section profiles of microstructured film of chimeric protein $Hmwb_5$-LmwCPR on silicone: (a) before treatment, (b) after treatment with a solutions of cytochrome b_5, and (c) after treatment with a solutions of cytochrome b_5 and NADPH-cytochrome P450 reductase.

reductase and (or) of their hydrophilic domains. It is established that these proteins as microstructured monolayers are able to block further adsorption of individual full-length proteins containing the same fragments. A possibility of specific protein-protein interactions at the adsorption of cytochrome b_5 and NADPH-cytochrome P450 reductase in the areas of hydrophilic silicone, not blocked by a monolayer of a chimeric protein is proved. Formation of protein complexes by means of such interaction is possible only at the certain orientation of the molecules, which is determined by the sequence of adsorption of proteins on the surface.

EXPERIMENTAL

Chimeric proteins $Hmwb_5$-LmwCPR (full-size cytochrome b_5 and hydrophilic domain of NADPH-cytochrome P450 reductase), $Hmwb_5$-HmwCPR (full-size cytochrome b_5 and full-size NADPH-cytochrome P450 reductase), $Lmwb_5$-LmwCPR (hydrophilic domain of cytochrome b_5 and hydrophilic domain NADPH-cytochrome P450 reductase), as well as cytochrome b_5 and NADPH-cytochrome P450 reductase were obtained by heterologous expression in the *E. coli* cells, and then isolated and purified to homogenic state using affinity chromatography (metal affinity chromatography on Ni-IDE Sepharose 6B for cytochrome b_5 and affinity chromatography on 2'5'-ADP Sepharose 4B for flavoproteins). The purity of proteins was estimated using SDS-electrophoresis and spectrophotometry.

For the preparation of the protein solutions phosphate buffer (pH ~7.4), Na-phosphate buffer (pH ~7.0), or bidistilled water were used.

For polydimethylsiloxane (PDMS) stamps a mixture was used of commercial prepolymer and Sylgard 184 catalyst (Dow Corning, Midland).

Silicon substrates were cleaned in a mixture of concentrated sulfuric acid and 30% hydrogen peroxide in the ratio 7:3 at ~70°C for 10–15 min, followed by five washing with distilled water.

Monomolecular protein films were isolated by adsorption from solution on the surface of the solid substrate or polymer stamp followed by washing with phosphate buffer (pH ~7.4) and distilled water, and dried under nitrogen.

Microstructured protein films were prepared by the technique [10]. The modified relief polydimethylsiloxane microstamp was brought into contact with the surface of the substrate in order to localize the transfer of the film of studied proteins from one surface to another in the places of contact of the stamp and the sample. As a master was used a calibration grid of atomic-force microscope TGZ3 (step 3 μm edge height 540±2 nm). For preparing a stamp, the prepolymer and

catalyst were mixed in mass ratio of 10:1, the mixture was deaerated with a water-jet pump for 15–20 min, then applied as a thin layer (~0.5 mm) on the surface of the master. Then termopolymerization was carried out at a temperature 100±5°C for 20 min. After cooling to room temperature, the stamp was separated from the master and either used within a day or stored in ethanol to prevent the polymer sweating on the stamp surface.

The AFM images of the surface were obtained in air with a Nanoscope IIId microscope (Veeco, USA) equipped with a D-scanner, in contact mode. We used 100 and 200 μm cantilevers Nanoprobe of Si_3N_4 with a constant elasticity 0.12 and 0.36 N m^{-1}, respectively. The impact of the needle on the sample in contact mode was 5 to 10 nN. The horizontal sweep frequency at obtaining the images ranged from 1 to 5 Hz.

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