===== **CELL BIOPHYSICS** =====

The Formation of Ordered Structures of Bacterial Porins in a Lipid Bilayer and the Analysis of Their Morphology by Atomic Force Microscopy

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Abstract—Ordered nanostructures of porin from the outer membrane of *Yersinia pseudotuberculosis* (YpOmpF) were formed in two ways: from proteoliposomes and by direct protein reconstitution in the pre-deposited phospholipid bilayer on mica surface. The morphological analysis of the structures was performed by atomic force microscopy. It was shown that the efficiency of formation, the degree of homogeneity, and the size of porin domains substantially depend on the experimental conditions and the presence of lipopolysaccharide in a porin sample or in the bilayer. It was found that using proteoliposomes resulted in formation of the aggregates of porin nanodomains on the mica surface, with uneven distribution in the bilayer and quite different size ranges (50−250 nm). In the case of direct reconstruction of porin, it was shown that a decrease in pH of the solubilizing buffer promotes the inclusion of a sufficiently large amount of protein as homogeneous domains with an average size of 35−40 nm but does not lead to the formation of extended nanostructured regions in the bilayer. The most efficient incorporation of porin into the lipid bilayer with the formation of clusters of tightly packed protein domains was achieved using a porin sample in combination with peptidoglycan and lipopolysaccharide, which this protein is tightly bound to in the native bacterial membrane.

In recent years there has been a considerable progress in applications of nanomaterials for biosensors, including those based on protein structures [1, 2]. At present, integral membrane proteins with a strong β-barrel structure are increasingly often considered in making biological nanopores. The main advantages of protein nanopores are the knowledge of their precise structure at atomic resolution and the possibility of introducing functional groups into strategic positions inside the channel. Such structures are widely used in nanobiotechnology and nanomedicine [3]. They include biogenic nanoparticles for therapeutic or diagnostic purposes, vectors for targeted drug delivery, native, or artificially constructed chemical and biological sensors. Nevertheless, in spite of the advantages of protein nanostructures and the examples of their successful development, one of the permanent limitations is the absence of methods for obtaining a rigid protein "framework" that would maintain its functionality within a wide range of environmental conditions.

Among the promising candidates for constructing biological nanopores are nonspecific porins channels of the outer membrane of Gram-negative bacteria that provide the transport of low-molecular weight substances across the outer membrane. Porins are β-barrel integral membrane proteins that form oligomeric structures (most often, trimers) in the native membrane. They consist of antiparallel amphiphilic beta-strands with hydrophobic and hydrophilic amino acid residues exposed to the lipid bilayer and the interior of the barrel, respectively [4]. The amphiphilic nature of porin molecules allows them to be easily incorporated in the lipid bilayer [5]. In the presence of phospholipids, porins can spontaneously form nanostructures due to protein–protein interactions [6]. Such a form of self-organization of two- and three-dimensional structures from nanosized components is considered as a simple and economically profitable method of obtaining nanomaterials. The choice of a detergent and a lipid is of great importance for obtaining two-

Abbreviations: LPS, lipopolysaccharide; AFM, atomic force microscopy; OG, β-D-octylglucoside.

dimensional crystals of pore-forming proteins; at the same time, the chemical structure of these components determines the parameters of ordered protein structures [7, 8].

The present work was aimed at developing approaches to the production of ordered nanosized structures on the basis of OmpF porin from the outer membrane of *Yersinia pseudotuberculosis* and elucidating the role of bacterial membrane components, lipopolysaccharide (LPS) and peptidoglycan, as vectors for self-assembly in a lipid bilayer on a solid surface. Comparative analysis of the morphology of nanostructures obtained under different conditions was performed by atomic force microscopy (AFM). The results are expected to be used for creating porin-based biomatrices that are potentially applicable to the development of complex inorganic nanostructural compositions for different purposes.

MATERIALS AND METHODS

Cultivation of bacteria. *The strain H-557 of Y. pseudotuberculosis* 0:1B serovar was used in this work. Microorganisms were cultivated at 4°C in a nutrient broth (Makhachkala, Russia) in 1-L flasks under conditions of intensive aeration for 5 days.

Isolation of porin and its complexes with peptidoglycan. The peptidoglycan−porin complex was obtained by bacterial cell extraction with the ionic detergent sodium dodecyl sulfate according to Rosenbusch [9]. The LPS level in the sample was measured as described in [10]. The isolated porin with LPS admixture was obtained as described in [11]. The protein was purified from LPS by the treatment with 30% sodium dodecyl sulfate solution as described in [12]. The resultant porin sample did not contain LPS. It was further purified by gel filtration in the presence of a 1% solution of nonionic detergent β-D-octylglucoside (OG) in Tris-HCl buffer (0.03 M, pH 7.5). The resultant porin−OG sample contained a trimeric porin and was homogeneous according to the data of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Obtaining large carboxylfluorescein-loaded unilamellar liposomes. Negatively charged liposomes were obtained as follows: a mixture of lecithin (30 mg), cholesterol (12.5 mg) and dicetyl phosphate (1.8 mg) was dissolved in 0.3 mL of chloroform, evaporated, and dried under a vacuum for 3 h. The remainder was dissolved in 0.3 mL of diethyl ether, followed by the addition of 50 µL of water and 50 µL of 0.2 M sulforhodamine B solution in 0.1 M Na_2CO_3. The mixture was shaken and exposed to ultrasonic bath treatment (Elmi, Latvia) for 10 min at 5°C and then to vacuum evaporation for the complete removal of the ether. To remove unbound sulforhodamine B, the liposome suspension was washed with 0.03 M Tris-citrate buffer, pH 5.5 or 8.0, precipitated by centrifugation at

25000 g, and suspended in the appropriate buffer. Liposomes of a uniform size were obtained by the ultrasonic bath treatment (Elmi, Latvia) of both portions for 10 min at 5°C and 10-fold filtration through a polycarbonate filter membrane with a pore size of 200 nm (Nucleopore, United States). The size of the liposomes was controlled by dynamic light scattering with a ZetaSizer Nano ZS (Malvern, Great Britain) at 25°C (scattering angle, 173° laser wavelength, 633 nm).

Recording changes in liposomal membrane permeability. The pore-forming activity of the protein was determined by recording the variation in liposomal membrane permeability. First, the background fluorescence was determined by adding 50 µL of liposome suspension to 120 µL of 0.03 M Tris-citrate buffer, pH 5.5 or 8.0. Next, 20 µL of porin solution exposed for 5 h in the respective buffer was added to the resultant liposome suspension and fluorescence change was recorded for 30 min. The maximum fluorescence under complete lysis was determined by adding 100 µL of 10% sodium dodecyl sulfate solution to the liposome suspension. The percentage of specific release of marker (SRM) was calculated by the formula:

$$\% \text{ SRM} = F_{exp} - F_{back} / F_{max} - F_{back} \times 100\%,$$

where F_{exp} is the fluorescence after the addition of protein, F_{max} is the fluorescence after the complete lysis of liposomes, and F_{back} is the fluorescence without the addition of protein.

Obtaining samples for atomic force microscopy. Proteoliposomes were obtained by long-term (48-h) dialysis of a mixture of a porin−OG sample solution (1 mg/mL) and synthetic phospholipid didodecyl phosphatidylcholine at a ratio of 1/1000 (w/w) against Tris-HCl buffer (0.03 M, pH 7.5) [13]. The suspension of proteoliposomes washed from unbound protein was applied to a freshly cleaved mica plate (10 × 10 mm).

The supporting lipid bilayer was formed on mica from didodecyl phosphatidylcholine and the porin−OG and peptidoglycan−porin samples were reconstructed in the presence of 0.3 M nonionic detergent dodecyl maltoside as described in [14]. There were two variants of incorporation of the porin−OG sample in the pre-formed bilayer. One variant was reconstruction of 50 µL of porin (100 µg/mL) in Tris-HCl buffer (0.03 M, pH 7.5) in the bilayer without LPS. In the second variant, 50 µL of LPC (100 µg/mL) from *E. coli* 055 (Sigma, United States) was layered onto the lipid bilayer and washed from unbound LPS, followed by introduction of the protein in acetate buffer (0.05 M, pH 5.5). The peptidoglycan−porin sample suspended in acetate buffer (0.05 M, pH 5.5) was applied to lipid bilayer without LPS pre-formed on the mica.

Atomic force microscopy. Images of porin preparations applied to a freshly cleaved mica plate were obtained with a Bioscope Catalist atomic force micro-

scope (Bruker, United States) in the contact mode in the buffer using the Bruker's proprietary technique in the Scan Asyst mode. The experiment was conducted with a ScanAssyst-Fluid cantilever (k ~0.7 N/m; tip radius, <10 nm) designed for working in liquid media. The clamping force was preset within a range of 120 pN. The nanostructural organization of porin in the samples was analyzed as described [13].

RESULTS AND DISCUSSION

The nonionic detergent OG was chosen to solubilize the porin from *Y. pseudotuberculosis* because it has been shown previously that the spatial structure of OG protein in solution is highly similar to its structure in the native membrane [15]. The following two techniques were used to obtain ordered nanostructures from the porin−OG sample on the mica surface. One of them was to obtain proteoliposomes from the porin solubilized in detergent in the presence of synthetic phospholipid: didodecyl phosphatidylcholine. This phospholipid was chosen because the thickness of the hydrophobic region of the lipid bilayer obtained from this phospholipid is commensurable with the height of the β-barrel of outer membrane porins [16] according to the published data, which provides maximum efficiency in the reconstruction of membrane proteins [17]. The other technique consists in preliminary formation of a supporting bilayer on the mica surface, followed by incorporation of porin molecules in detergent solution into the bilayer and washing of the sample surface with a buffer without the detergent [14].

Figure 1a shows that porin nanoclusters obtained by the former technique have a high level of aggregation and a considerable spread in diameter (50−250 nm). According to the scale of staining intensity in Fig. 1a, protein aggregates protrude by 14−28 nm above the lipid matrix. They are unevenly incorporated in the phospholipid bilayer formed during the interaction between the lipid of proteoliposomes and the flat-surface substrate. The great difference between the sizes of observed nanoclusters is most likely due to the fact that they are a result of spontaneous aggregation of protein molecules during the long-term dialysis of porin−detergent−phospholipid mixture for obtaining proteoliposomes. It should be noted that the tapping-mode scanning of the surface of this sample showed a considerable shift of the AFM image (as is shown in the lower part of Fig. 1a), indicating the mobility of protein aggregates within the bilayer formed during proteoliposome fusion.

The preliminary formation of a supporting phospholipid bilayer by way of liposome fusion on mica provides rather high surface coverage. Nevertheless, in the right-hand part of Fig. 1b there is a bright area that apparently corresponds to nonfused liposomes. As one can see from Fig. 1c, the lipid bilayer is 4−6 nm in height, which correlates with the thickness of the phospholipid bilayer. The darker areas on the 3D

(a)

(b)

(c)
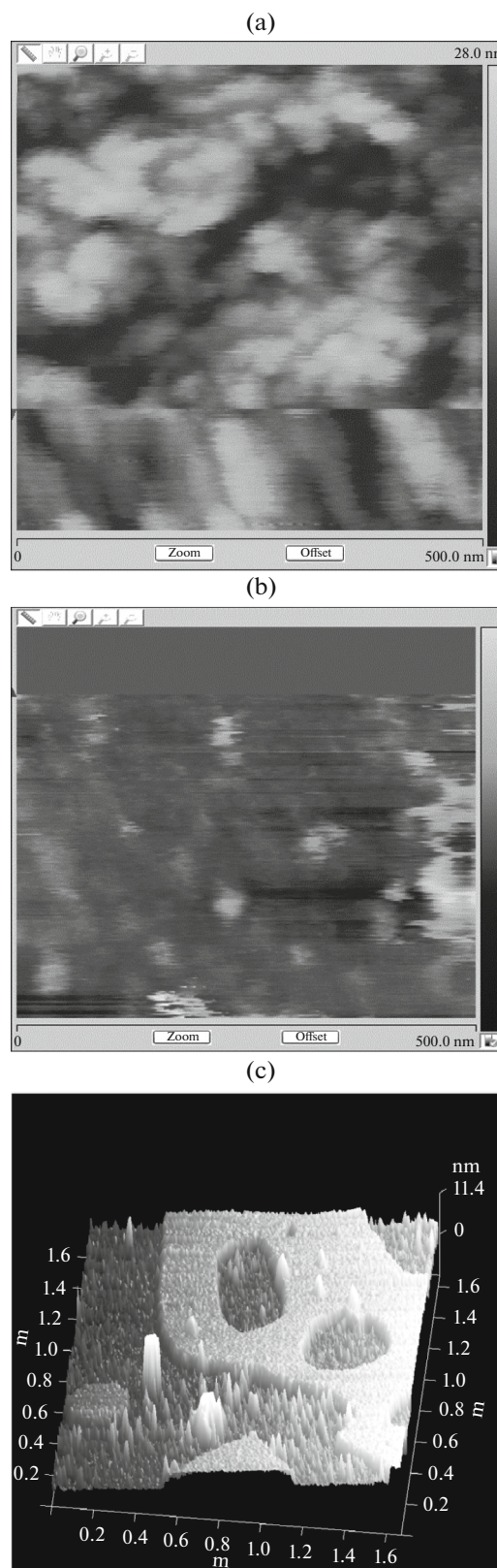
Fig. 1. The AFM images of porin−OG nanostructures obtained by the application of proteoliposomes to the mica (a), the incorporation of the protein into the supporting phospholipid bilayer preformed on the mica (b) and its 3D image (c). The samples were scanned in 0.03 M Tris-HCl buffer, pH 7.5.
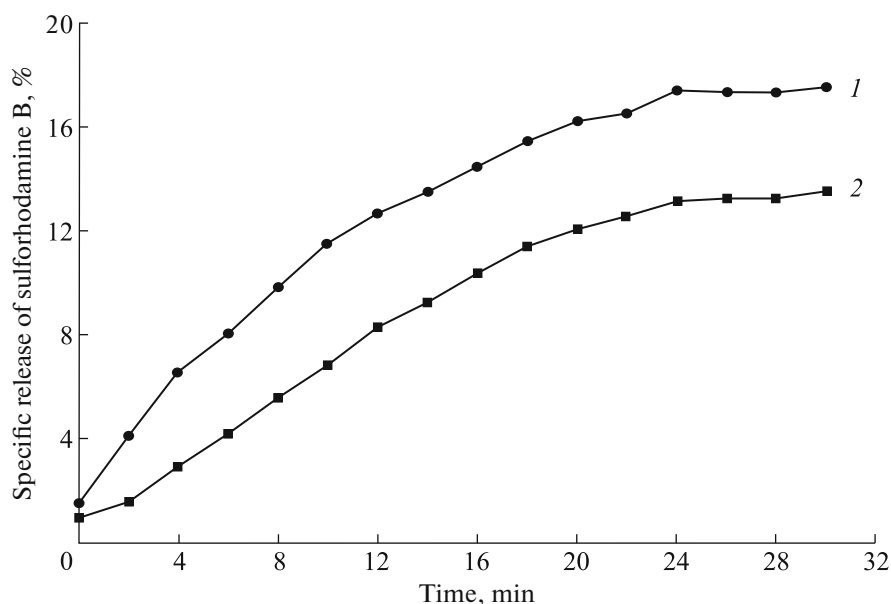
Fig. 2. The kinetics of sulforhodamine B release from negatively charged liposomes on the addition of the porin−OG sample at different pH values of buffer solution: curve (*1*) pH 5.5; curve (*2*) pH 8.0. The Y axis: specific release of sulforhodamine B.

image probably correspond to the partially removed bilayer that can be destabilized by detergent solution at the stage of protein incorporation.

Thus, the direct method of porin reconstruction in the pre-formed supporting phospholipid bilayer does not lead to the formation of long sections of 2D crystals of the protein and only single inclusions can be seen. This might be caused by two factors. First, porin samples were solubilized in the buffer with pH close to the neutral value (pH 7.5) and this is probably not the optimal condition for effective reconstruction of porin in the lipid bilayer. Secondly, the experiment was conducted with highly purified protein samples without admixtures of other outer membrane components, primarily LPS. As is known, porins in the native membrane exist in complex with LPS; these two major components of bacterial membranes are linked to each other by a tight but noncovalent bond. Some data demonstrate that the interaction between LPS and porin plays a key role in protein assembly in the outer membrane of bacterial cells during biosynthesis [18]. In addition, it has been shown that LPS considerably influences the efficacy of porin incorporation in the lipid bilayer [5]. With regard to the pH of the buffer solution, we previously have shown that the change in pH value of the medium has a considerable effect on the molecular conformation of porin from *Y. pseudo-tuberculosis*, its spontaneous reconstruction in the bilayer and, accordingly, manifestation of its functional activity [19].

In view of the above, the next step of the research was to determine the optimal conditions for incorporation of porin sample into the lipid bilayer. We used the method of fluorescent probe efflux from the nega-

tively charged liposomes during protein incorporation for this purpose. The concentration of sulforhodamine B-based fluorophore inside liposomes in the initial suspension was higher than the concentration of its self-quenching. Pore formation in the liposomal membrane upon the addition of porin sample was accompanied by the efflux of sulforhodamine B-based fluorophore to external solution and, accordingly, in the noticeably higher intensity of its fluorescence.

The liposomes obtained from lecithin, cholesterol, and dicetyl phosphate proved to be quite resistant to the OG-containing buffer where porin was solubilized. As follows from Fig. 2, the graphs of sulfor-hodamine B release from the liposomes during incorporation of porin in the buffers with acidic and weakly alkaline pH values are substantially different. The most effective porin incorporation was observed at pH 5.5 of the solution. Taking these findings into account, further experiments were performed with protein samples solubilized in an acidic buffer solution (acetate buffer, pH 5.5).

The effect of LPS on formation of the ordered structures of porin from *Y. pseudotuberculosis* was experimentally verified using two porin samples with different levels of LPS: (1) the isolated porin without LPS (porin−OG) and (2) the peptidoglycan−porin complex with an LPS level of 8−10%. In the former case (Fig. 3), the porin−OG sample was introduced into the LPS-containing lipid bilayer pre-formed on the mica.

Figure 3 shows that the amount of porin incorporated into the bilayer under these conditions (in the presence of LPS and in a weakly acidic medium) is much higher compared to protein reconstruction in a
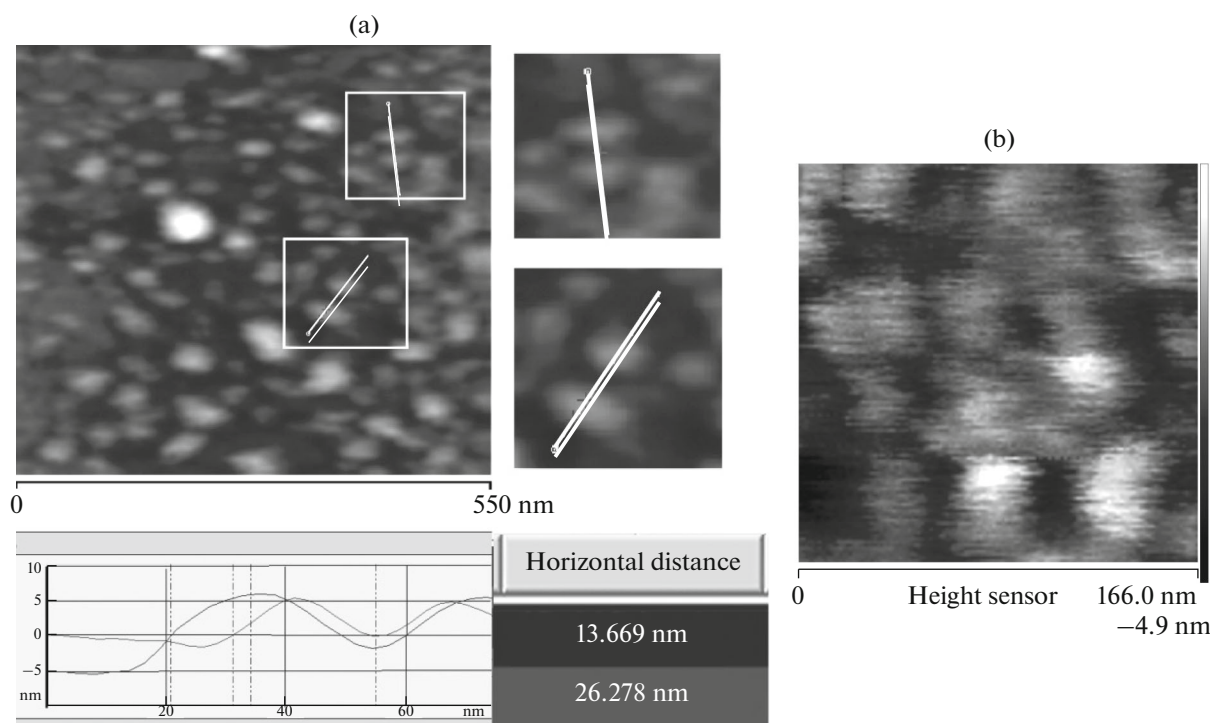
Fig. 3. The AFM image of the porin−OG sample in the supporting LPS-containing phospholipid layer at different scales: 0.5 μm (a) and 0.16 μm (b). The lower part of the figure (a) shows sample surface profiles along the lines in the frames used to calculate the mean particle diameter. The height of domains protruding above the bilayer surface was determined by the colorimetric scale to the right from the Fig. (b). The samples were scanned in 0.05 M acetate buffer, pH 5.5.

weakly alkaline medium. Regularly located porin domains with an average diameter of 35−40 nm and a height of 6−10 nm occur on the bilayer surface.

In the latter case (Fig. 4), LPS was not introduced into the bilayer because, according to the published data [10], the peptidoglycan−porin complex contained no more than 10% LPS, which is in agreement with the data on the peptidoglycan−porin complex from *E. coli* [9]. The amount of porin in the complex with peptidoglycan is more than 4 times higher than the level of peptidoglycan [9]; at the same time, the protein retains all of its bonds with peptidoglycan and LPS. As a consequence, the spatial organization of the porin molecule most closely matches its native conformation in the membrane. This has been demonstrated by the high functional activity of such complexes as detected by the bilayer lipid membrane technique [20]. In view of the above, as well as of the fact that peptidoglycan bound to porin makes up a rigid "framework" of bacterial membrane, we suppose that incorporation of the peptidoglycan−porin sample into the bilayer can lead to formation of the most ordered homogeneous protein clusters on the mica surface.

As follows from Fig. 4, the surface of a peptidoglycan−porin sample in the lipid bilayer is presented by rather long sections that consist of a set of rosette-like clusters (shown by ellipses) of similar size (approximately 80 nm). However, the length and packing density of these nanostructural formations vary between different sections of the bilayer surface. The above clusters are an aggregate of separate domains. Their lateral sizes were measured by the most typical minimal details of the AFM image (shown by square frames). The distances between the minimums on the chart of the linear scan profile are 20 nm on the average. We assume that this value corresponds to the size of the protein domain incorporated into the bilayer. Such an assumption is based on the fact that the porin molecule, which consists of three subunits, has a lateral size of 7−8 nm as determined by AFM [21]. In view of the above, it can be supposed that these porin domains contain more than one molecule of trimeric porin, which is well in agreement with the published data. According to the modern concepts, porin can exist in the native membrane as hexa- and nanomers [22]. On the other hand, the size of the porin domain can visibly increase due to the effect of lateral broadening associated with the finite radius of curvature of the cantilever.

Thus, as a result of these studies, we have tested different approaches to the formation of ordered nanostructures of OmpF porin from the outer membrane of *Y. pseudotuberculosis* in the presence of phospholipid bilayer. Analysis of the morphology of AFM images has shown that isolated porins can be spontaneously incorporated into a phospohlipid bilayer with the for-
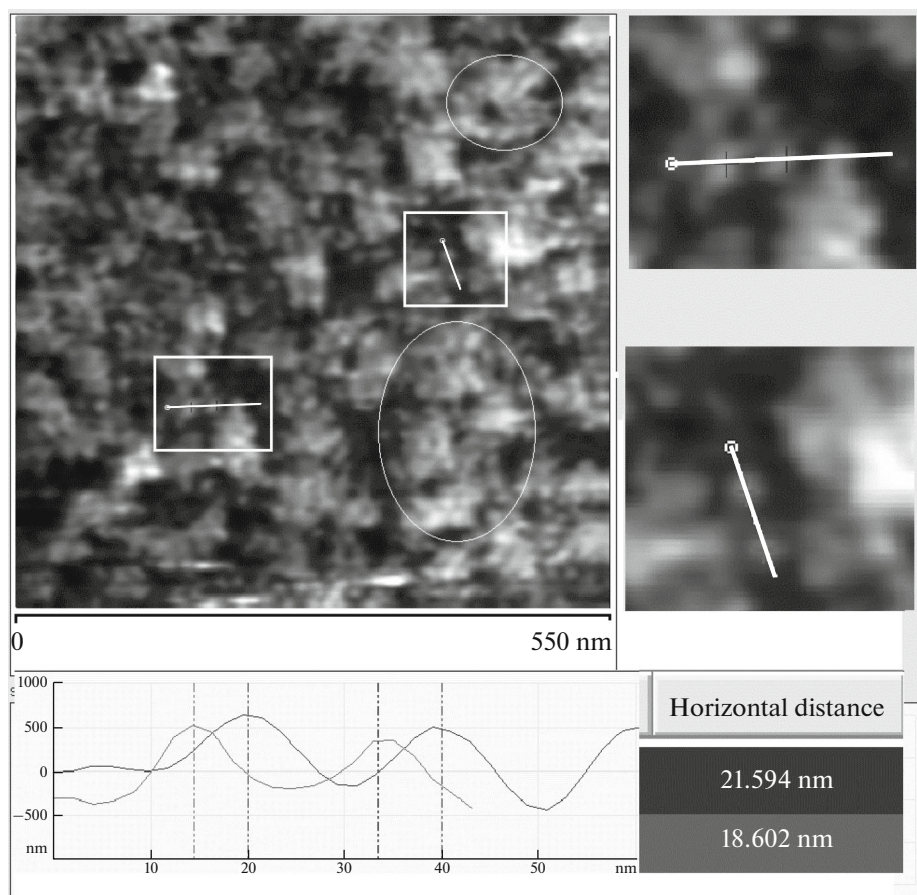
Fig. 4. An AFM image of the peptidoglycan−porin complex in the preformed lipid bilayer without LPS. Ellipses show the porin clusters consisting of protein domains. Below, is a sample surface profile along the lines in the frames used to calculate the mean sizes of porin domains.

mation of nanodomains. At the same time, the level of efficacy and the size of porin domains substantially depend on experimental conditions. It has been shown that the number of protein domains incorporated into the bilayer increases at higher pH values of the solution. It has been shown that LPS is an effective vector for porin incorporation at acidic pH values. The second component of the bacterial outer membrane (peptidoglycan, which porin is linked to in the native state) makes a maximum contribution to the formation of long ordered protein domains.

It should also be noted that the peptidoglycan−porin complex is enriched in porin and does not contain other membrane proteins, because its production is accompanied by almost complete purification from other protein components of bacterial outer membrane [9]. In view of the above, the approach described in this work can be quite promising for obtaining nanostructures on the basis of functionally active membrane proteins, compared to using intact bacterial cells as a matrix [23].

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies involving animals or human participants performed by any of the authors.

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