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AFM study of complement system assembly initiated by antigen-antibody complex

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Abstract: The shape and size of complement system C1 components assembled on a SiO_2 surface after classical activation by antigen-antibody complex was determined by tapping mode atomic force microscopy (AFM). The SiO_2 substrate was silanized and bovine leukemia virus proteins $gp51$ were covalently bound to the SiO_2 substrate. Self-assembly of complement system proteins was investigated by AFM. Uniform coating of silanized surface by $gp51$ proteins was observed by AFM. After incubation of $gp51$ coated substrate in anti-$gp51$ antibody containing solution, Ag-Ab complexes were detected on the substrate surface by AFM. Then after treatment of Ag-Ab complex modified substrate by guinea-pig blood serum containing highly active complement system proteins for 3 minutes and 30 minutes features 2-3 times and 5-8 times higher in diameter and in height if compared with those observed after formation of Ag-Ab complex, were observed respectively on the surface of SiO_2. This study revealed that AFM might be applied for the imaging of complement system assembly and provides valuable information that can be used to complement other well-established techniques.

1 Introduction

Biological self-assembling systems are governed by nanoscale processes that evolved over millions of years, but only the most successful examples of biological systems have been surveyed. In order to understand the functioning of biological systems, knowledge about the character of biomolecular interactions and structure of formed complexes is crucial. The convergence of nano-scale science with modern biology, medicine and bioanalytical chemistry is a promising trend [1]. Nanotechnology provides the tools and technology platforms for the investigation and transformation of biological systems, and biology offers inspiration models and bio-assembled components to nanotechnology. Emulating the concepts and principles of biology has led to the controlled self-assembly of biomaterials. Self-assembly and self-organization have inspired ideas for advanced bioengineering methods at the nano-scale [2]. One of such unique self-assembling bio-nanomachines is a complement system. The complement system is the major effector of the humoral branch of the immune system [3] and plays an essential role in host defense against infectious agents and in the inflammatory process [4, 5].

The complement system is composed of over twenty proteins involved in a sequence of cascading reactions ultimately resulting in the elaboration of biologically active products and destruction of the cellular membranes [6]. The complement system may be initiated through the "classical pathway" by the binding of antibodies to cell surface antigens, through the "alternate pathway" by the presence of foreign cell surface components, such as polysaccharides, as well as antibody aggregates and throught the "lectin pathway" by the mannan binding protein [7, 8]. The initial step in the activation of the classical pathway involves interaction between the first component of complement, C1, and a number of receptor sites present on the immunoglobulin molecules forming an complexes [9]. Thus, the most important proteins for self-assembly of complement system are C1 proteins (C1q, C1s and C1r), which are involved in such general complex formation processes: the complexing of antibody with antigen induces conformational changes in the Fc portion of the antibody molecule that exposes a binding site for the C1 component of the complement system; C1 (approximately 900 KDa) exists in serum as a macromolecular complex consisting of C1q (400 KDa) and two molecules each of C1r (168 KDa) and C1s (83 KDa), held together in a complex ($C1qr_2s_2$) stabilized by Ca^{2+} ions. The C1q molecule is composed of 18 polypeptide chains that associate to form six collagen-like triple helical arms, the tips of which bind to exposed C1q-binding sites in the C_H2 domain of the antibody molecule. Each C1r and C1s monomer contains catalytic and interaction domains; the latter facilitates interaction with C1q or with each other. Each C1 molecule must bind, via its C1q globular heads, to at least two Fc sites for a stable C1-antibody interaction to occur [10].

Following initial activation, various complement components interact, in a highly reg-

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ulated enzymatic cascade, to generate reaction products that facilitate antigen clearance and generation of inflammatory response. The complement reaction products amplify the initial antigen-antibody reaction and convert it into a more effective defense mechanism. Finally, the terminal components of the complement system generate the membrane-attack complex [11]. The set of proteins necessary to build a functioning complement system is present in the blood of all mammalians. However, self-assembling of this system starts only in the case if particular antigen-antibody complex is formed on the surface of cell membrane where antigens are exhibited. Also, circulating Ag-Ab complexes are able to fix complement system proteins. Due to complement receptors, such immune complexes are delivered to the organs of the monocyte-phagocytic and reticuloendothelial systems. This process can be affected by environmental contamination [12]. The complement system of guinea-pig is especially active and is often used as a model system for investigations of complement system function [13].

The function of specific actions in complement system is under intensive investigations: function of complement proteins [14–16]; structure and function of complement activity controlling protein was predicted [5]; influence of various factors on regulation of complement-mediated cytotoxicity [17]; inactivation of complement system [18]; etc. Because of the complexity of the complement system, the real structural model that might be obtained by AFM will be useful for detailed investigations of this complex system. Applicability of AFM for investigation of other biomacromolecules-based complex systems has been demonstrated: for structural analysis of the reaction center light-harvesting complex I [19], observation of Chitosan-induced restructuration of a mica-supported phospholipid bilayer [20], study of single protein based systems [21], in vitro reconstitution of fibrillar collagen type I assemblies at reactive polymer surfaces [22], visualization of band-like cellulose assemblies produced by bacteria [23], characterization of surface-immobilized layers of intact liposomes [24], morphology study of starch, amylose, and amylopectin films [25] etc.

We believe that atomic force microscopy might be very useful for investigations of complement system assembly and the aim of this study was to show that complement system assembly might be investigated by means of tapping mode AFM.

2   Experimental part

2.1   Chemicals

Bovine leukemia virus (BLV) proteins $gp51$ as well as the specific serum containing anti-$gp51$ antibodies were obtained from 'Biok' (Kursk, Russia). Ethanol, 3-aminopropyl-thriethoxysilane (APTES) (Sigma, St.Louis, USA) was used for SiO_2 substrate preparation. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimid (EDC) (Sigma, St.Louis, USA) was used for protein carboxyl group activation. Blood serum of guinea-pig containing active complement system proteins and BLV not infected cattle blood serum were obtained from the Institute of Immunology of Vilnius University (Lithuania, Vilnius).

2.2 Silanization of SiO$_2$ substrate

SiO$_2$ substrate and test-tubes were washed by ethanol and dried by heating. SiO$_2$ substrate was placed into test-tubes with APTES and heated for 1 hour at 90 °C. After washing with water, SiO$_2$ substrate was prepared for protein immobilization.

2.3 Immobilization

50 mg/ml solution of *gp*51 in 0.9 % NaCl was prepared and activated with EDC solution for 1 hour. Freshly prepared surface of 8 Å SiO$_2$ was exposed to activated antigen *gp*51 molecules for 7 h. Antigen solution used for immobilization gives an approximate area density of 40-50 molecules per μm^2. Active groups that were not engaged in formation of covalent bonds were deactivated by 0.1 M glycine solution, pH 7.0. The surface was then rinsed with water.

2.4 Preparation of samples for AFM study

Before each AFM measurement the surface of modified 8 Å SiO$_2$ was rinsed with water and dried using a flow of dry Argon gas. To study antigen-antibody interaction, the surface of 8 Å SiO$_2$ covered with immobilized antigens was exposed to antibody containing solution for 30 min., rinsed, dried, and studied by AFM. To study antigen-antibody-complement system interaction the surface containing antigen-antibody complexes was exposed to guinea-pig serum rich with active complement system proteins for 1 h and then it was rinsed, dried and probed by AFM.

Before each AFM-imaging samples were rinsed in 0.1 M glycine buffer, pH 7.0, for 5 min. to prevent non-specific interactions and then thoroughly rinsed in deionized water to prevent crystallization/aggregation of glycine extent. Then samples were dried by flowing argon gas.

2.5 AFM imaging

High resolution tapping mode Atomic force microscope (AFM) measurements were performed with a home-built AFM interfaced with NT-MDT Corp. (Zelenograd, Moscow, Russia) control electronics and contact mode measurements were made by AFM "Q-Scope 250" Quesant Instrument Corporation (Agoura Hills, USA).

The dry samples were investigated by AFM in the contact and tapping mode in a range of scan lengths from 5 μm to 1 μm. The commercial Si cantilevers NSG11 series (length 100 μm and width 35 μm) with a force constant 11 Nm^{-1} and tip curvature 10 nm and resonanse frequency 255 kHz (NT-MDT) were used for tapping mode measurements and soft cantilevers with force constant 0.25 Nm^{-1} were used for contact mode measurements.

The slides of 0.5 cm^2 surface area of 8 Å SiO$_2$substrate received from AIXTRON AG (Aachen, Germany), were used as substrates for AFM investigations.

AFM images are presented in two formats: large format (Fig. 1 A,B,C,D) representing characteristic image of 5000 × 5000 nm area of modified surface and detailed format (Fig. 3 A,B,C,D) with height width diagrams where characteristic features are analyzed.

For all AFM images 200 × 200 pixels image resolution was applied, scanning rate 10 μm/s.

3   Results and discussion

The immunoreagents bovine leukemia virus (BLV) protein gp51 and polyclonal anti-gp51 were selected for current experiments because of high affinity of these reagents [26] and high stability of gp51/anti-gp51 complex [27, 28]. Moreover, BLV infections are still frequent and detection of those infections as well as effective treatment methods are important [29]. The experimental concept of the presented experiment was based on the measurement of radius and height of features observed by scanning probe microscope, because the height and radius of these features was dependent on the complex size of immobilized/formed macro molecular species.

AFM imaging was performed in dry state which is a cheaper and more basic alternative compared to AFM imaging in liquid environment [30]. AFM images of SiO$_2$ substrate surface before and after silanization process seem to have no significant differences in morphology (Fig. 1A) or any significant change in roughness presented in corresponding histograms (Fig. 2A), therefore silanization insignificantly changes those properties of the surface in applied resolution scale. After covalent attachment of gp51 proteins, the differences in morphology (Fig. 1 B) and roughness (Fig. 2B) of substrate were detectable at our AFM equipment resolution scale. From the image presented it clearly seems that 180-230 nm diameter and 7-9 nm height (Fig. 3A) features appeared on the surface of silica. It is estimated that observed features (Fig. 3A) are bovine leukemia virus protein gp51 and/or its complexes covalently immobilized on the surface of silica substrate [31, 32]. The results obtained illustrate that success of covalent protein immobilization can be estimated by AFM.

Optimal incubation time (30 min.) of gp51 modified surface with anti-gp51 Ab was determined in previous experiments [33]. The AFM images (Fig. 1C, Fig. 3B) registered after 30 min incubation of gp51 modified silica in solution containing anti-gp51 antibodies showed significant differences in morphology (Fig. 1C, Fig. 3B) and roughness (Fig. 2C) if compared with roughness (Fig. 1B, Fig. 3A) and morphology (Fig. 2B) registered before this treatment or obtained by equally treated control SiO$_2$ surface which was not covered by gp51 (data not shown). In images of mica surface investigated by AFM after exposure of gp51 modified SiO$_2$ to anti-gp51 Ab containing solution (Fig. 1C, Fig. 3B) two distinct populations of features with height-radius corresponding to those of gp51 and antigen-antibody (gp51/anti-gp51) complex are observed. If gp51 modified surface was exposed towards solution containing set of other antibodies but not containing any anti-gp51 observed features (Fig. 3E) were similar to those observed on the gp51 modified SiO$_2$ surface before treatment with antibody containing solution. Expected gp51/anti-

gp51 complexes were larger in height-radius almost twice if compared with immobilized gp51 proteins (Fig. 1B, Fig. 3A). The width-height of gp51/anti-gp51 complex was approximately twice higher what represents the range of Ag-Ab complex dimensions [34–38]. It allowed to distinguish between immobilized antigens and antigen-antibody complexes formed. The formed features allow us to conclude that we observe the antigen-antibody docking reaction, or the appropriate lack thereof, between single antigen molecules immobilized on the surface and single or multiple antibody molecules. However, the yield of such formations (surface area covered by 7-9 nm height features as presented in figure 2A was compared with area covered by 10-20 nm height features was measured and compared) not exceeded 7-15 % if compared with suspected gp51 immobilization yield, the main reason for it might be improper orientation of epitopes present in gp51, and this is in good agreement with the results obtained during the AFM detection of Ag-Ab complexes [39]. Since a high number (over 85 %) of immobilized antigen was not engaged in the formation of antigen-antibody complex we conclude that not all antigen molecules are properly oriented and/or denatured and they are unable to bind with Fab sites of specific antibody.

When a surface without immobilized antigens was exposed to the same antibody containing solution no clear features similar to those presented in Figures 1C and 2B were detected. The AFM image was absolutely identical to the image of unmodified SiO_2 substrate surface because in the absence of immobilized antigen specific affinity interaction and strong binding of any proteins to the surface was not reliable, not specifically adsorbed proteins were removed by 0.1 M glycine buffer, pH 7.0.

After 3 min. treatment of silica substrate modified with gp51/anti-gp51 Ab complex in the complement protein containing solution, 40-70 nm diameter and 30-40 nm height structures appeared on the surface (Fig. 1D and Fig. 3C). Height histogram (Fig. 2D) derived from figure 1D shows appearance of higher features if compared with those presented in figures 2A,B,C derived from figures 1A,B,C correspondingly. No similar structures were observed if unmodified silica substrate or silica modified just with gp51 was treated by the same complement system protein containing solution for the same period.

A significant increase in geometrical dimensions of the detected structure of previously formed Ag-Ab complex might be the result of self-assembly of a number of C1 components during early stage of complement system self assembly initiated by antigen antibody complex.

After 1 h treatment of silica modified with gp51/anti-gp51 Ab complex in the complement protein containing solution, sparse 500 nm diameter and almost 400 nm height structures appeared on the surface (Fig. 1E and Fig. 3D). Appearance of significantly higher features was confirmed by appearance of significantly higher structures in corresponding histogram (Fig. 2E). We believe that those structures were based on complement system proteins self-assembled on the basis of Ag-Ab complexes, while no such structures were detected if just gp51 modified silica substrate or unmodified silica surface was treated by the same complement system protein containing solution for the same

period.

After exposure of antigen-antibody complex containing surfaces to complement system containing solution we observed features larger in lateral dimension as well as in height than either *gp*51 or antigen-antibody complexes. The image appears to show complex formation, what was expected. The area density of the features is about 5-7 times smaller if compared with the density of antigen-antibody complexes, and about 20-25 times smaller if compared with the density of immobilized *gp*51; it allows us to suppose that some spatial factors are crucial for the formation of antigen-antibody-complement complexes. We favor the notion that the relatively large antigen-antibody complexes may exist in a wide range of orientations and conformations all of which are not optimal for antigen-antibody-complement complex formation. Moreover, since the mixture of anti-*gp*51 antibodies is polyclonal, several antibodies can bind respectively to different epitopes of *gp*51 and such complex might be inconvenient for the formation of antigen-antibody-complement components complex. Tapping mode phase images (Fig. 1E) clearly show at least two distinct populations of features with different rigidity that are forming complement system protein complex/complexes. This fact illustrates that different proteins in the complement system posses different rigidity and might be distinguishable based on this property by AFM phase imaging.

4　Conclusions and future developments

Our experiments demonstrate that AFM is usable for observing biological molecular processes like antigen-antibody docking and even for the formation of larger biological structures. However, from the AFM data presented in this study it is still not possible to clearly identify separate proteins, since the detection was performed in at least a partially dried state and the protein complex appeared in an almost globular shape.

The results presented encourage a detailed AFM study of complement assembly by application of purified complement system proteins.

Acknowledgment

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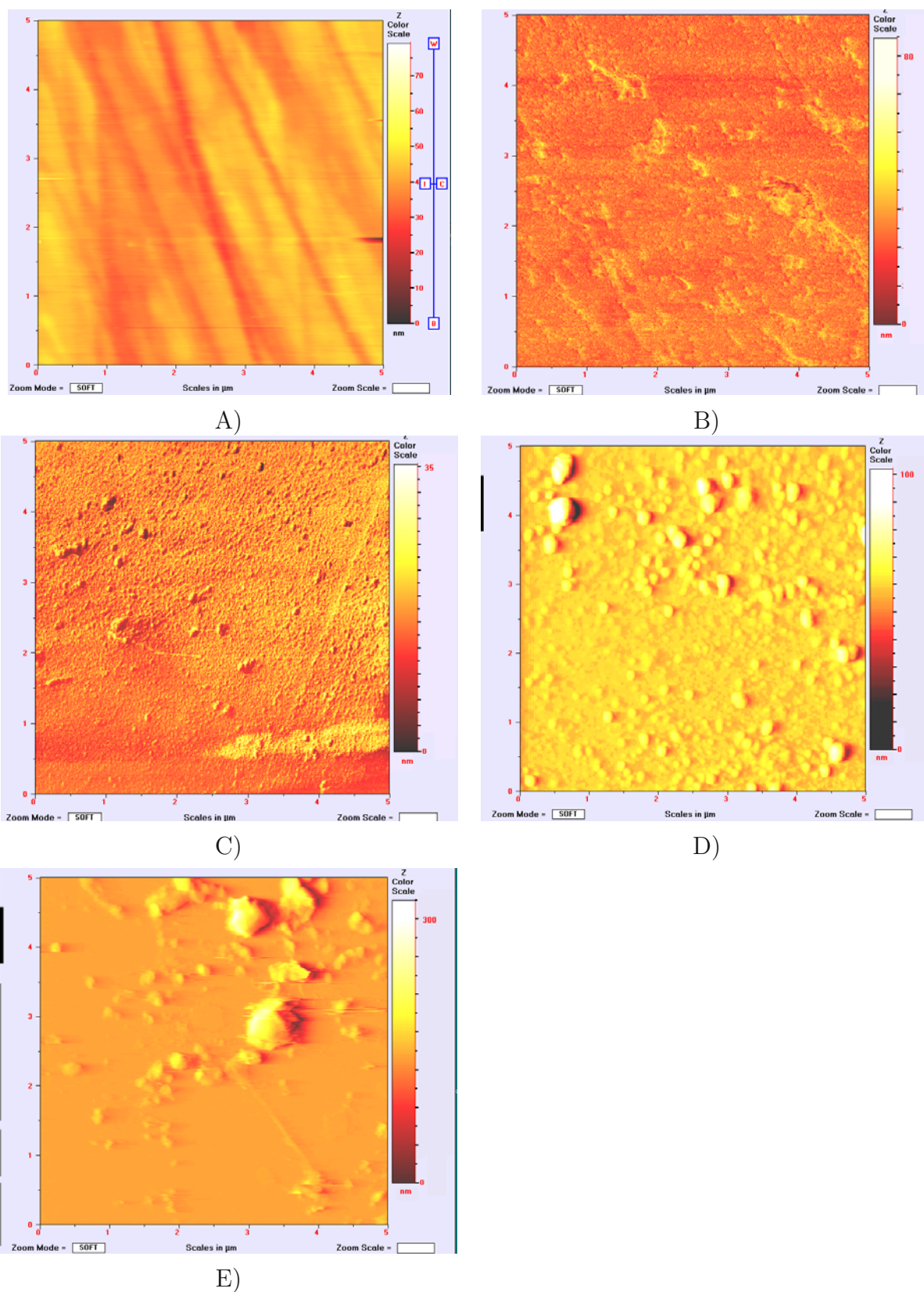
A)

B)

C)

D)

E)

Fig. 1 Tapping mode AFM images of (A) silanized SiO$_2$ and the same surface consecutively treated with: (B) *gp*51 for 2 h; (C) anti-*gp*51 for 30 min; (D) active complement system proteins for 3 min; (E) active complement system proteins for 60 min. Images covers area of 5000 × 5000 nm.
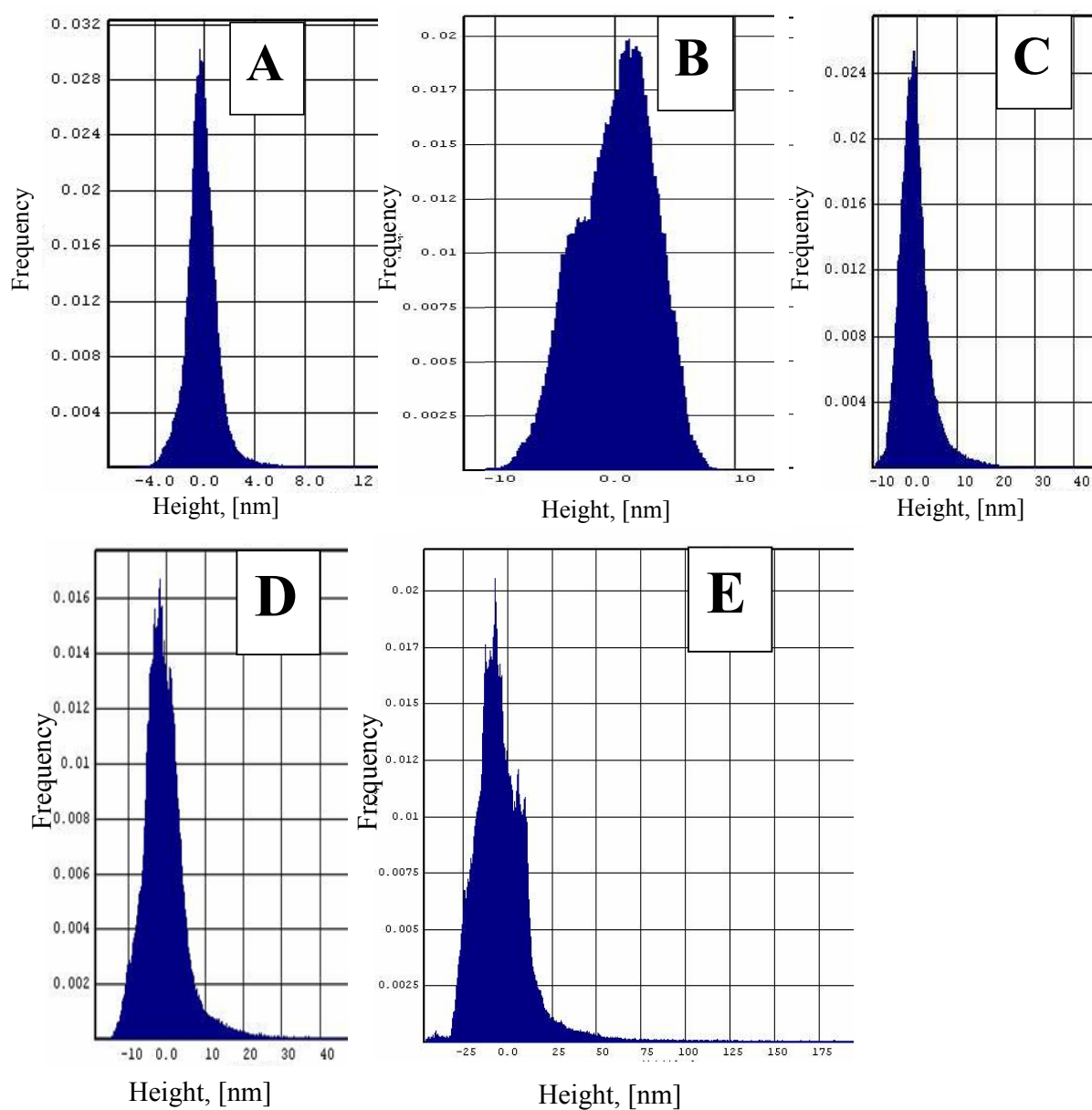
Fig. 2 Frequency diagrams of tapping mode of corresponding AFM images presented in Fig. 1. of (A) silanized SiO_2 and the same surface consecutively treated with: (B) *gp*51 for 2 h; (C) anti-*gp*51 for 30 min; (D) active complement system proteins for 3 min; (E) active complement system proteins for 60 min. Images covers area of 5000×5000 nm.
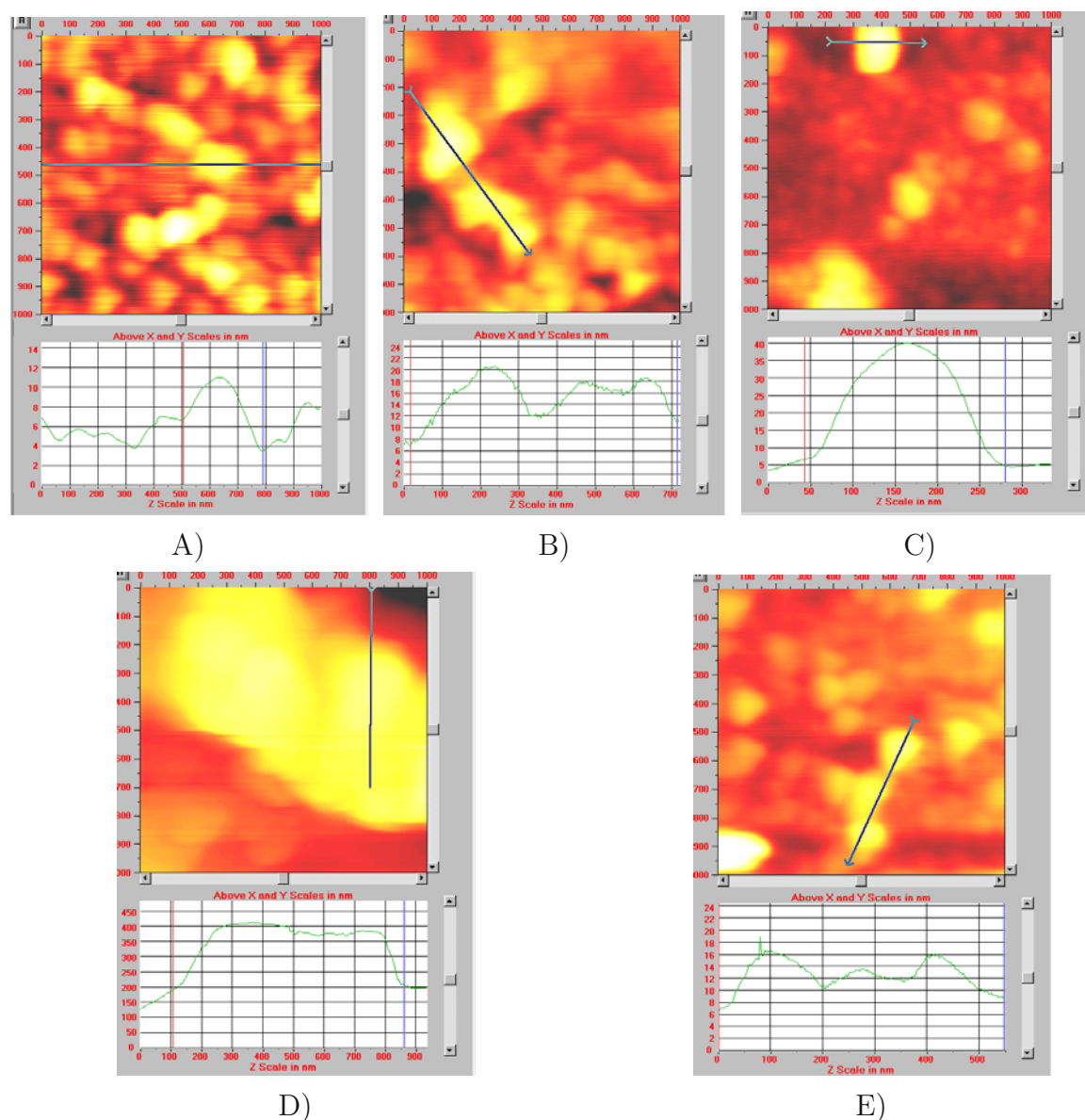
A)   B)   C)

D)   E)

Fig. 3 Height-width study of typical features observed by tapping Mode AFM image of silanized SiO_2 surface consecutively treated with: (A) gp51 for 2 h; (B) anti-gp51 for 30 min; (C) active complement system proteins for 3 min; (D) active complement system proteins for 60 min. Images cover area of 1000 × 1000 nm. Height profiles are shown in the lower panels for the black lines in the corresponding image; (E) control experiment silanized SiO_2 treated by gp51 for 2 h and BLV not infected bovine blood serum (not containing anti-gp51) for 30 min.