Patterns in biopolymers and other biological systems as observed by scanning probe microscopy

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SUMMARY: High resolution scanning probe microscopy images of DNA and RNA molecules, tobacco mosaic virus particles, growing lysozyme crystals, and bacterial cells are shown. The main advantage of scanning probe microscopy lies in the visualization of dynamic processes in biological systems in environmental conditions, e.g. in liquid media. Some results obtained during observation of lysozyme protein crystal growth in solution and bacterial cellular wall degradation due to lysozyme treatment are presented.

Introduction

Scanning probe microscopy (SPM) opens new possibilities in the investigation of biopolymers and biological objects. It provides new methods for molecular biology, microbiology and molecular medicine. The visualization of biological objects is the most intriguing application of SPM. Since 1995 our group has been engaged in the SPM study of nucleic acids, proteins, biomembranes, bacteria, cells of plants, animal and human cells. We have pioneered in the SPM imaging of RNA-protein complexes and different viral particles, living vector vaccines used in practical medicine. Here we present a review of recent results achieved at Scanning Probe Microscopy Joint Group of Moscow State University and Advanced Technologies Center (Moscow, Russian Federation).

Experimental technique

During the two last decades scanning probe microscopy and mainly atomic force microscopy has proved to be become a practical tool for the surface characterization at molecular and

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atomic level with a variety of practical applications. The present experimental studies of biological objects were performed using MultiMode AFM Nanoscope-3 (Digital Instruments Inc.) in air and liquid environments. FemtoScan software was used for image processing and filtering[1]. Recently we have implemented FemtoScan-Online AFM for real-time measurements and data acquisition from any remote computer via Internet. This apparatus was used in Open Learning program "From atoms and Molecules to Living Cells" recently launched for student education at Moscow State University. To our opinion this microscope also may be of definite interest for microbiological research centers for monitoring dynamic processes in living systems, for example, providing *in situ* studies of bacterial cells in solutions and monitoring their response to different chemical and medical reagents.

Nucleic acids

DNA was the first biomacromolecule visualized using AFM[2-5]. Still AFM imaging of DNA in air yields somehow similar results to that obtained by electron microscopy. Static images of DNA on mica substrates gives better resolution in electron microscopy, and DNA decoration with metals or carbon is not a restriction in this method while studying immobile objects. However it is not possible to apply electron microscopy for the study of study of DNA conformational changes in solutions and this was done with much success in our many other SPM groups. Typical AFM image of DNA molecules on mica substrate is shown in Fig.1.

At present time there is just a few results concerning the visualization of another nucleic acid - RNA. There are at least two reasons for that. First of all DNA extraction and purification meet more problems than in the case of DNA. Another problem arises during RNA immobilization on a flat substrate: RNA molecules do not adsorb to clean mica surface without any chemical treatment of mica. Mica surface modification with KCl, $MgCl_2$ or BAC (benzydimethylalkyl ammonium chloride) used in our experiments[6] slightly increases the overall substrate corrugation height similar to that seen in AFM studies of DNA. The visible in AFM height of RNA molecules was in the range 0.3-1.5 nm for both contact and tapping modes. To be sure that what we are seeing in AFM are really RNA molecules we perform visualization of: 1) TMV (Fig. 2a), which are composed of RNA and protein coating, 2) partially uncoated virus particles with protruding parts of RNA (Fig. 2b), and 3) pure

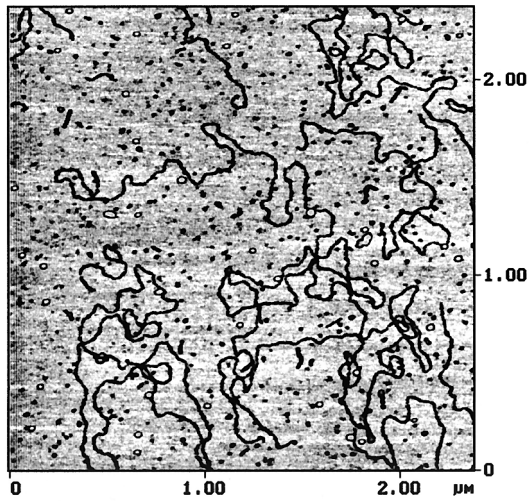

Fig. 1: AFM image of DNA on mica substrate. Contact mode, image size *2,3 x 2,3* μ^2.

RNA molecules (Fig. 2c). The RNA molecules shown in Fig 2c are spread in one direction by hydrodynamic flow during washing using distilled water.

Proteins

As a fact AFM is not able to resolve inner structure of proteins besides some individual cases when submolecular resolution was achieved. Still a new information concerning protein adsorption to surfaces, its distribution on the substrates, incorporation in organic film and biomembranes can be obtained. The fulfilled investigation of proteins, protein-membrane[7)], protein-polyelectrolyte complexes[8)] have a direct interconnection with the development of high sensitive biosensors and new medical reagents.

AFM gives new advantages over other high resolution technique in the observation of dynamic processes involving protein molecules: aggregation, crystallization and thin film formation.

We have applied AFM for the in-situ observation of lysozyme crystal growth[9-11)]. The structure of surface defects and kinetics of kinks and steps movement are studied. The

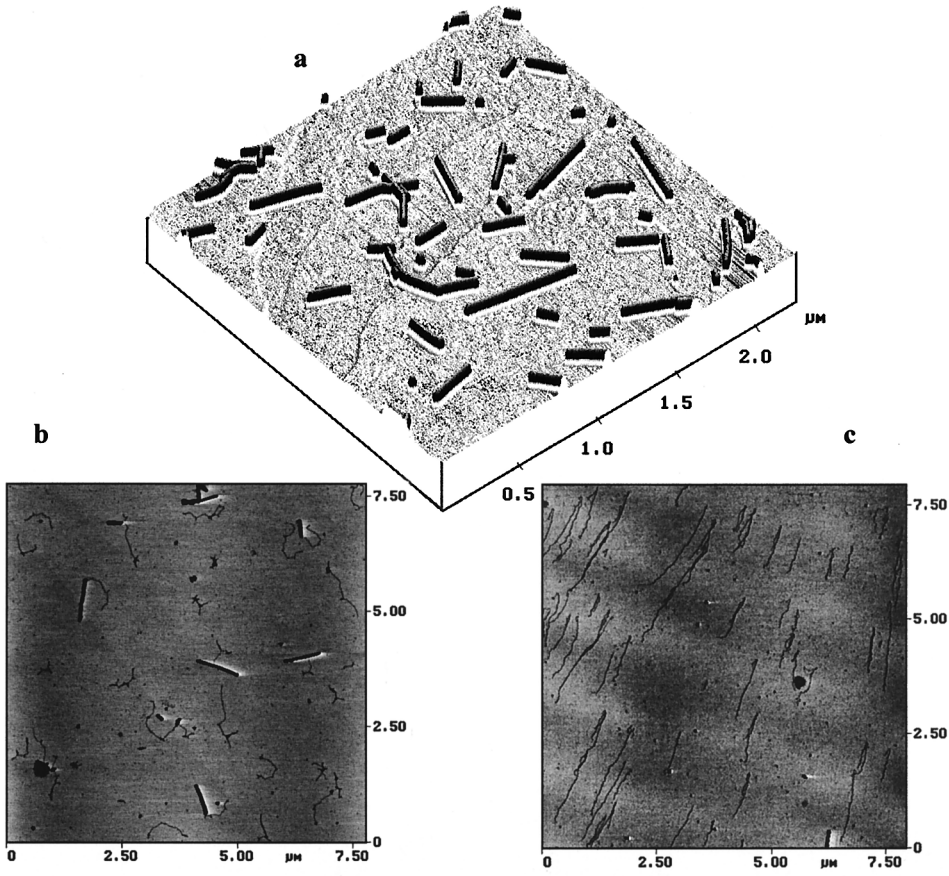

Fig. 2: (a) Tobacco mosaic virus (TMV) particles on graphite substrate. Image size 2,5×2,5 μ^2.
(b) Partially uncoated TMV particles with protruding parts of RNA molecules on mica substrate. Image size 7.5 x 7.5 μ^2.
(c) RNA macromolecules released from protein coating using dymethylsulfoxide cloride.
Mica substrate. Image size 7.5 x 7.5 μ^2. (For details see ref. 6)

following main results are achieved. We have obtained the molecular resolved images of protein crystal growing in solution (Fig. 3a). It was found that screw dislocations on a crystal surface are the main sources of growth (Fig. 3b). The most developed (010) face was investigated in the constant force mode. The molecular resolution was achieved at small forces lower than 10^{-10} N. A small increase of applied force leads to the appearance of

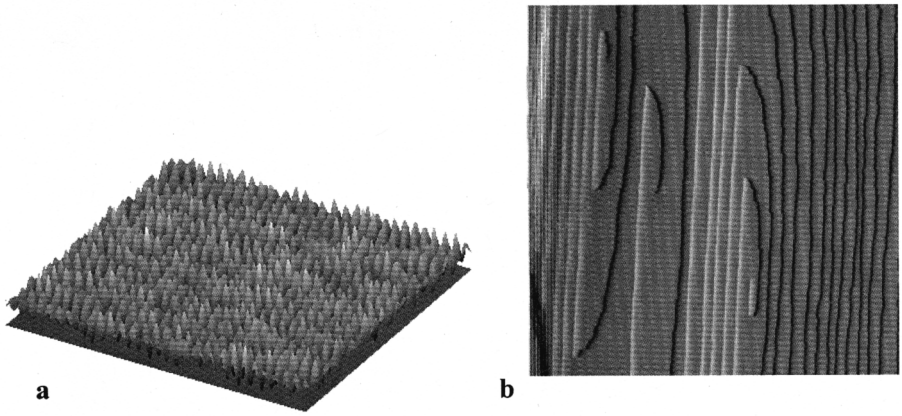

Fig. 3: (a) AFM image of (010) face of lysozyme crystal with molecular resolution, image size 130 x 130 nm^2 (left). (b) Screw dislocations on the (010) face, image size 11 x 11 μ^2.

different contrast of neighbor rows of unit cells in *a*-direction as it is shown in Fig. 4. The force-induced contrast indicates on the possible surface reconstruction on the (010) face of lysozyme crystal of orthorhombic modification.

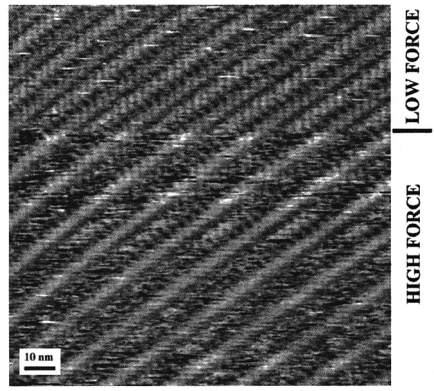

Fig. 4: The increase in force applied to the crystal surface (from 10^{-10} N to $1.5x10^{-10}$ N)leads to the changes in the observed image: the appearance of period doubling in a-direction on the (010) face. Image size 130 x 130 nm^2.

Bacterial cells

We have performed AFM characterization of enterobacteria of different taxonomy groups[12-13] (*Escherichia coli*, *Klebsiella*, *Helicobacter pilory*, *Bifidobacterium*, etc.). In all the cases we use a simple procedure for the deposition of bacterial cells on the substrate. Freshly grown

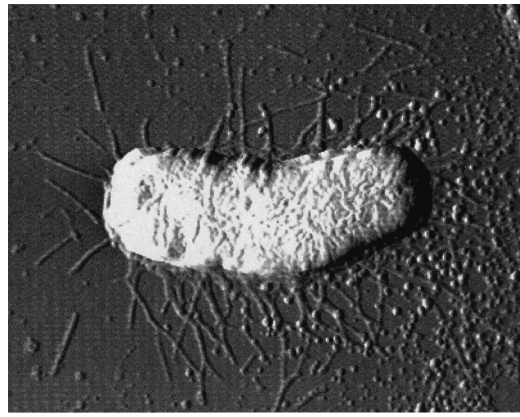

Fig. 5: Image of *E. coli* JM109 bacteria on mica substrate. Image size 3 x 2,5 μ^2.

bacteria were placed in distilled water for a short time by a loop at a concentration about 10^9 in ml. Then a drop of the solution was put on the surface of freshly cleaved mica. The first observation was carried out 10-15 min after the deposition. The image of *Escherichia coli* bacteria is shown in Fig. 5. The control experiments with E.coli have shown that the images are quite stable in a year or two after the deposition. It was found that bacterial cells tend to form mainly close-packed monolayer films (Fig. 6). Multilayer bacteria films are not merely observed. The above shown images of bacteria were obtained at air, this means in dry conditions. It is worth noting that the cells are still alive and after putting in proper conditions the cells are able to grow and divide.

The sample preparation is very simple so AFM has remarkable advantages in comparison to electron microscopy and AFM may be regarded as practical tool for microbiological applications. The comparative SPM analysis revealed structural difference between natural

enterobacteria and artificially produced living vaccines using modern methods of gene exchange[12].

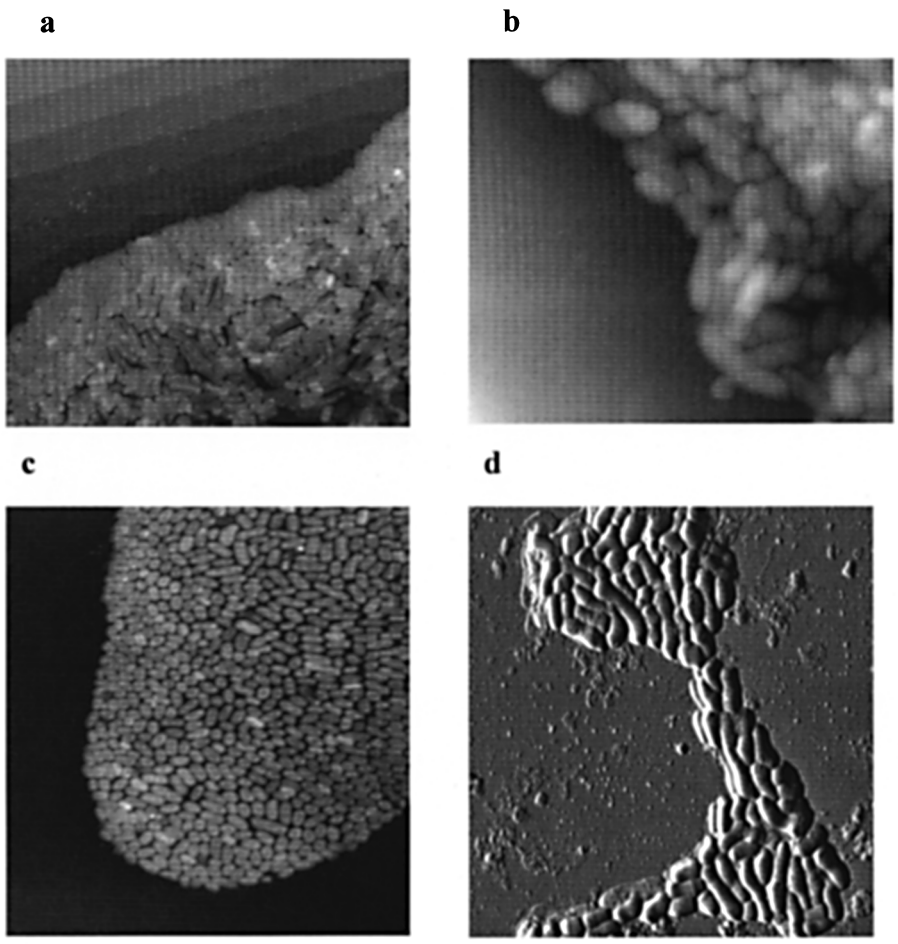

Fig. 6: Monolayers formed by different bacteria: *Escherichia coli* (a), *Arthrobacter globoformis*, (b), *Klebsiella pneumoniae* (c), *Helicobacter pylori* (d).
Image sizes: 31 x 28 μ^2 (a), 13 x 14μ^2 (b), 40 x 43 μ^2 (c) and 14 x 14 μ^2 (d). The bacteria are deposited on freshly cleaved mica.

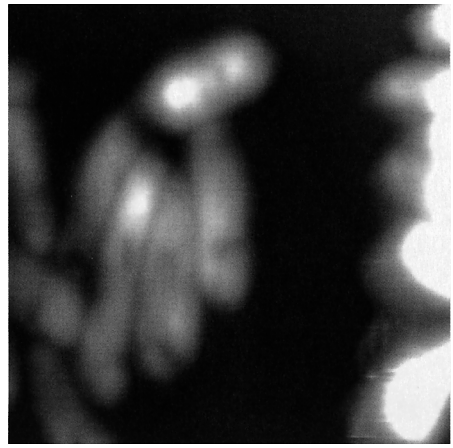

Fig. 7: AFM image of *Escherichia coli* bacteria in water. Image size 13 x 13 μ^2.

Bacterial cells have a rigid cellular wall. This makes possible to observe them using AFM in contact mode in a wide range of forces (from 10^{-10} up to 10^{-8} N) without any visible damages of its surface. We have performed comparative study of *Arthrobacter globoformis* bacteria in two different forms: vegetative and mummy[14]. It turned out that vegetative bacteria are able to withdraw as high forces as 10^{-6} N, while such a force leads to the destruction of mummy bacteria. It is consistent with the prediction that cellular wall of mummy is more fragile than that of vegetative bacteria.

The bacteria images obtained in liquid environment reveal less contrast than in air. Some of the cellular surface features obtained in air are not observed in water. The surface of bacteria becomes much more smother. Fig. 8 shows AFM image of *E.coli* bacteria on mica substrate imaged in water. The influence of the substrate on the morphology of bacteria is most probably negligible due to low adhesion of bacteria to mica surface in water. The low adhesion can be proved by the fact that the bacteria can be removed from the surface by the AFM tip when the applied force to the bacteria from the tip is about 1 nN. The AFM observation of drastic changes of bacteria shape in buffer solution after addition of lysozyme

is performed recently in our group[15]). This is a well-known effect of lysozyme on bacteria, which leads to the formation of spheroplast — bacteria with partially destroyed cellular wall.

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