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High resolution imaging of IgG and IgM molecules by scanning tunneling microscopy in air condition

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KEYWORDS

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Abstract A scanning tunneling microscope is a powerful tool for obtaining micrographs from conductive and semiconductive materials. The imaging technique has recently been improved for microscopy of nanostructured biomaterials on highly ordered atomic surfaces. We describe, here, high resolution imaging of individual IgM and IgG using a scanning tunneling microscope (Nama-STM) in air condition. The biomolecules were immobilized on the surface of Highly Ordered Pyrolytic Graphite (HOPG). Obtained micrographs could reveal structural details of immunoglobulins G and M on the atomically flat surfaces. Obtained results confirmed that STM could be more useful than other microscopy techniques for the analysis of single biomolecules.

1. Introduction

Scanning Tunneling Microscopy (STM) is a powerful technique for studying conductive and semiconductive surfaces [1]. The microscope could also be useful in studying atoms and molecules adsorbed onto the conductive and semiconductive materials [2]. The technique offers very high resolution micrographs from the nanomaterials without using elaborated sample preparations or requiring ultra-high vacuum equipment [3,4].

The principle of STM is based on the quantum mechanical phenomenon, so-called 'tunneling effect' [4,5]. When a sharp needle (i.e., tip) is placed less than 1 nm distance from a conductive surface (i.e., sample) and a voltage applied between them, the electrons can tunnel between the tip and the sample through the narrow vacuum barrier [6]. As the tunneling current exponentially varies within the tip-sample distance, a small change of less than a fraction of the atomic length can be detected in this space [6,7].

High-resolution imaging of nanostructured materials, especially sensitive and soft biomolecules, demonstrates a great challenge in biology [8]. The structure of individual biomolecules has been previously studied at nano-scaled resolution by high-resolution transmission electron microscopy, X-ray crystallography, and atomic force microscopy [8–11]. These techniques require sample-preparation procedures, which potentially alter the sample nature [9]. For example, atomic force microscopy could be used for imaging of biomolecules under ambient or physiological conditions, but special precautions are required to prevent the tip from damaging or altering samples [10,11].

The ability of scanning tunneling microscopy for analyzing geometrical and electrical features of biomaterials has been improved for a wide range of some structurally well-characterized biomolecules, such as polypeptides, proteins, enzymes and DNA [12–16]. One of these structurally well-characterized proteins is immunoglobulin. Immunoglobulin molecules are generally a unit consisting of two identical light polypeptide chains and two identical heavy polypeptide chains. These are linked

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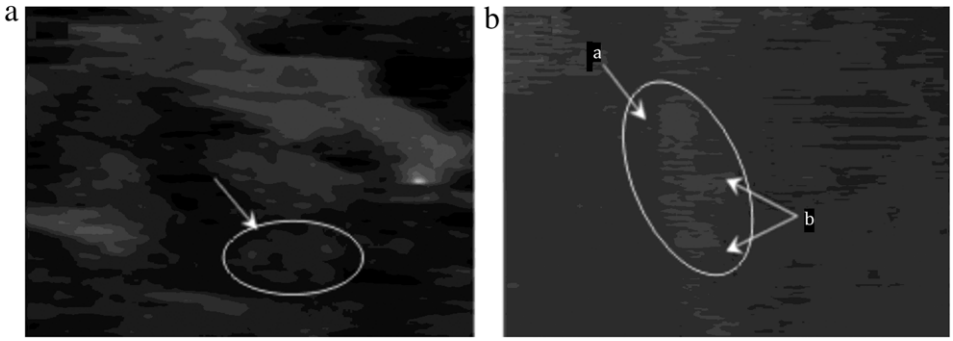

Figure 1: Image of a single antibody molecule without any filtering or coloring process after cropping. (a) IgM molecule with scan area 170 × 150 nm; (b) IgG molecule with scan area 30 × 25 nm. Note that 'a' represents F_c, and 'b' F_{ab} regions of a single antibody (IgG) molecule.

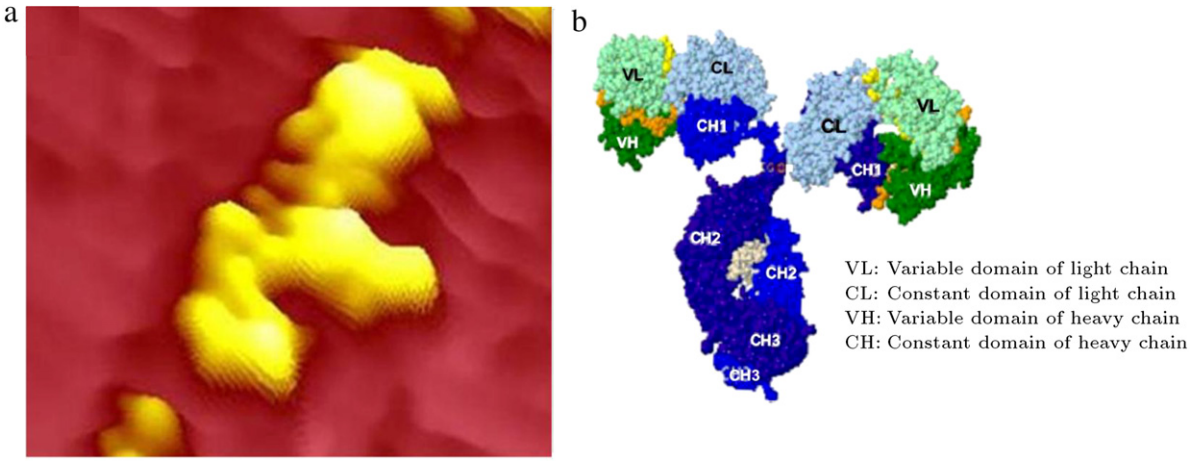

Figure 2: (a) 3D image of a single antibody (IgG) molecule after the filtering and coloring process, which shows orientation of this molecule after physical adsorption on the rigid surface from the hinge region imaged by NAMA-STM. (b) Graphical conformation of *Y*-shaped IgG (with kind authorization of Marie–Paule Lefrance, IMGT®, http://www.imgt.org).

together by disulphide bonds and the hinge region is a segment of the heavy chain between CH1 and CH2 domains. The three-dimensional morphology and size of immunoglobulins revealed the fact that the biomolecules may be disoriented when immobilized on the surface of rigid materials (such as HOPG) [17]. Here, we introduce a simple procedure for the single molecular imaging of IgG and IgM in detail, using scanning tunneling microscopy in air condition.

2. Materials and methods

2.1. Chemicals and instruments

General human IgG and IgM samples were purchased from Iran Avicenna Research Institute (http://www.avicenna.ac.ir). The samples were used without further purification. Deionized water (Milli-Q; Millipore) was used for the buffer preparation. The micrographs were obtained by NAMA-STM SS-1 (Nanotech System Corporation, Natsyco, Iran). HOPG was also prepared from the Nanotech System Corporation. Other chemicals were prepared from Sigma-Aldrich.

2.2. Sample preparation

Human IgG and IgM were diluted from original stocks in phosphate sodium buffer (0.12 mM NaH_2PO_4, 0.84 mM NaCl, 0.046 mM NaN_3, pH 7.4) and brought to a concentration of about 2 μg/ml. Then, 5 μl of diluted sample were dropped on the HOPG for 30 s at 50 °C to be dried [16].

2.3. Scanning tunneling microscopy

The specimens were scanned with NAMA-STM. STM tip was prepared mechanically using Remanium with 0.35 mm diameter (Dentaurum, Germany). All experiments were performed in air at room temperature with at relative humidity of ∼40%. The constant current mode was used to take images. The images were obtained with a scan rate of 2000 Hz and a current set point of around 0.1 V. The sample bias voltage for the images was 1 V, which during the scans was increased to 1.6 Volts gradually. During the scan, we also decreased the current set point from 0.1 nA to 50 pA. Rough data were first processed by using median (middle range) and low pass Gaussian (high rang) filters. Then, the coloring process was tested on the obtained micrographs for different levels [16].

3. Results and discussion

Figure 1 shows single IgM and IgG molecules before any graphical process. The images were obtained after drying on the surface of HOPG. The unprocessed images (Figure 1(a) and (b)) do not demonstrate the structural domains and they need some image processing to be resolved in detail.

Figure 2(a) shows the expected size of three lobs (two F_{ab} and an F_c) in IgG, which are in complete agreement with information from atomic force microscopy [18] and X-ray crystallography studies [19]. Figure 2(b) shows a representative

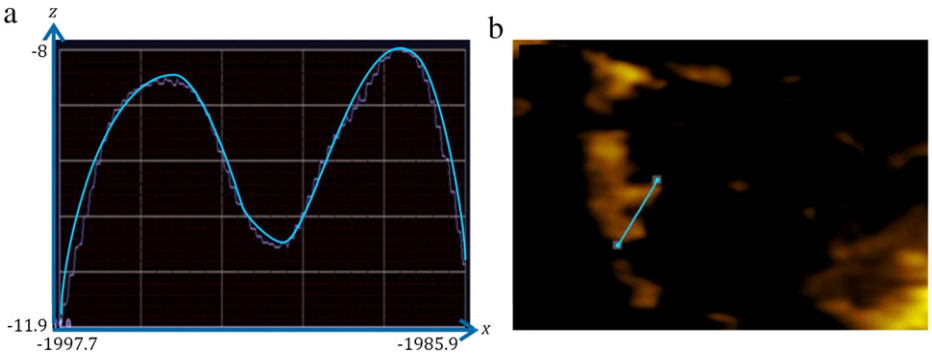

Figure 3: (a) Line profile of antigen binding site or F_{ab} regions. (b) 2D image of a single antibody (IgG) molecule after filtering and coloring process.

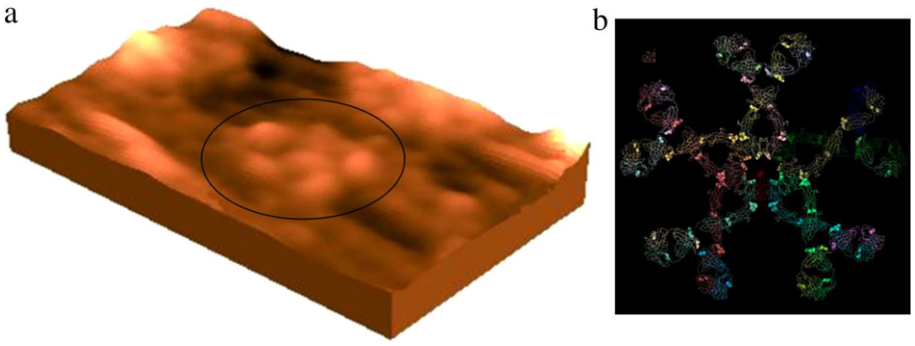

Figure 4: 3D image of a single antibody (IgM) molecule, imaged by NAMA-STM. (b) Standard configuration of human immunoglobulin *M* with pentameric domains [21].

of a single IgG molecule in its original condition after image processing. The image could be compared with the antibody in the solution phase with three dimensional aspects. Although possible tip contact with the sample during the scan may change the shape of the original molecule structure, applying different image processing and coloring filters could enhance the image quality brilliantly. For biomolecules with a *Y* shape, the small differences in the lateral dimensions of the F_{ab} or F_c domain by AFM, X-ray, and TEM were 8 nm and 6–7 nm, respectively [18]; however, our measurements were around 10–11 nm. These differences may be due to the drying process, as well as to the orientation of the molecule on the rigid surface, or even due to calibration errors of the instrument.

Paulo and Garcı´a used tapping-mode atomic force microscopy in both attractive and repulsive tip-sample interaction regimes [18]. Their images for the attractive interaction regime allow determination of the basic morphologies of the antibodies on the support. This regime was able to resolve the characteristic *Y*-shaped domain structure of antibodies and the hinge region between domains. They concluded that the imaging in the repulsive interaction regime was associated with the irreversible deformation of the molecules [19]. But, again, their image could not give full details about single antibody molecules.

Figure 3(b) shows the second step of the imaging process after applying some filters and also the coloring process of the image from the top view. The left-side image (Figure 3(a)) shows the line profile of F_{ab} regions, which also gives, roughly, the size of these two domains. The size of the molecule was measured around 37 nm.

Quist and colleagues reported the earliest image showing a *Y* shape [20]; however, the image did not resolve the separation between domains. The authors stated that only a very small number of molecules ($\ll$1%) showed the above morphology [18]. It seemed that biological structures under the pressure of the AFM tip could be easily deformed or even damaged. Even at sub-nanonewton probe forces, the pressure within the contact area might be many times the atmospheric pressure. The immediate effect of severe deformation, even without specimen damage, was the loss of surface features in the AFM. To overcome these difficulties and to improve the fidelity of the methodology, using cryogenic temperatures for AFM imaging has been proposed as a possible alternative [21, 22].

Figure 4 shows a single IgM molecule after image processing. IgM is a big molecule with a size of around 35 nm. The micrograph demonstrated the pentameric structure of the IgM molecule on a flat surface clearly.

Cryo-AFM images contained a conformation with a much higher center column and a diameter of 35–45 nm. Not only did a small minority show the flat pentameric form of 45–50 nm diameters, but also the small domain in the center appeared to be the *J*-chain. According to reports, for the purpose of a direct comparison with those obtained at cryogenic temperatures, no stable and reproducible images could be obtained. The major problem was that the IgM molecules were simply swept aside during scanning and broke into smaller pieces [23].

4. Conclusions

There are several SPM studies involving the imaging of antibodies [24–27], but only a few have produced images of antibodies with a standard shape. However, the molecules exactly appeared in the natural-shaped morphology. The difficulty in acquiring useful images of nanostructured biomaterials at room

temperature by SPM and especially STM, and the disappointing resolution achieved of most specimens can significantly be attributed to the softness of biological samples. The best images can be obtained at low current set points, around +0.1 nA, and tip voltages around +1 V. During the scan, the voltage should increase up to 1.6 V, and the current set point should be decreased up to 50 pA. This work made the STM tip far from the sample surface to prevent damaging the protein molecule, while the tunneling effect is occurred. In this way, it is possible to get images with a very high resolution from biomolecules. On the other hand, our results show more resolution of STM than of AFM, which is mostly used for such studies **in air condition**. Since the IgG molecule has a random rotational orientation on the surface, this feature is important for good STM imaging; however, the image processing (or filtering) also has a very important role.

It is clear that if somebody knows the molecule or has ideas about the orientation of this molecule after immobilization on the surface, a very sophisticated image can be obtained using STM. If the molecules are immobilized in such a way that the electrons are not able to tunnel through them, brilliant images could not be captured. Our experiments indicate that the immobilization process of biological samples is very important for suitable tunneling phenomena through nanostructured biomaterials on the hydrophobic surface of HOPG. In addition, less timing for water evaporation within a HOPG sampling could help biomolecules to be positioned on the surface in a better order than the results obtained when the samples had more time for the drying process at room temperature. In the other words, the suitable heating of HOPG before dropping the sample on it led to a shorter opportunity for the overcrowding and aggregation of biomolecules, and inhibits their positioning layer-by-layer at one point.

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