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Binding activity of patterned concanavalin A studied by atomic force microscopy

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Abstract

The mode of protein immobilization plays a crucial role in the preparation of protein microarrays used for a wide spectrum of applications in analytical biochemistry.

The microcontact printing technique was used to form a protein pattern using concanavalin A (Con A) since Con A belongs to a group of proteins widely used in analytical assays due to their selectivity as regards different kinds of carbohydrates.

Atomic force microscopy was used to image surface topography, delivering information about the quality of the protein pattern. The force spectroscopy mode was used to verify the functional activity of deposited proteins via determination of the forces of interaction between Con A and carboxypeptidase Y bearing carbohydrate structure recognized by Con A.

The calculated binding force between Con A and CaY was 105 ± 2 pN and it was compared with that measured for Con A deposited directly from the protein solution. The similarity of the value obtained for the interaction force was independent of the mode of protein deposition, thereby verifying that the microcontact printing technique did not influence the carbohydrate binding activity of Con A.

The correlation between the surface topography of patterned samples and adhesion maps obtained showed the possible use of AFM for studying the chemical properties of different regions of the micropatterns produced.

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1. Introduction

Despite the successes of modern pharmacology and surgery, an appropriate and correct diagnosis is still necessary to conquer a disease. Fast, easy, qualitative and inexpensive analytical methods are required for a wide spectrum of applications. Therefore, it is very much necessary to carry out several bioassays in parallel as well as there being a demand for integrity and miniaturization of biosensors. Both of the above requirements have led to interest in the fabrication of biomolecule patterns. In the last decade, significant achievements have been reported in the development of biomolecule microarrays and polymers used in biosensor production (Wang 1999, Bilban *et al* 2000, Heng and Snyder 2003, Ruano *et al* 2003, Petrenko and Sorokulova 2004, Adhikari and Majumdar 2004, Saxena and Malhotra 2003, Gerard *et al* 2002).

The immobilization of biological molecules on a sensor's surface is very much crucial for facilitation of biosensor production. Many methods have been developed to solve this problem and lots of them are now very well established. However, the protein immobilization still remains an important problem since protein functionality depends on the mode of protein translation on a substrate (Heng and Snyder 2003, Mirzabekov and Kolchinsky 2001).

There are many different ways of immobilizing proteins on hard substrates (usually glass, mica or gold surfaces). These techniques involve rather expensive methods based on photochemical and/or self-assembled monolayer techniques (Blawas and Reichert 1998, Morgan *et al* 1995). However, there is an alternative technique used for protein immobilization called microcontact printing (Bernard *et al* 1998, Kane *et al* 1999, Bernard *et al* 2000), where a polymer stamp with a given pattern is used to transfer molecules onto a substrate. The stamp pattern is reproduced on the substrate. This method has found applications in patterning of proteins (Garrison *et al* 1999), in cell growth on well defined surface structures (Scholl *et al* 2000, Balaban *et al* 2001, Csucs *et al* 2003, Cuvelier *et al* 2003, Chang *et al* 2003) and in production of patterns using alkanethiols (Libioulle *et al* 1999, Fujihira *et al* 2001) or other polymers (Csucs *et al* 2003). The mode of the pattern formation should protect the biological activity of the proteins, which can be verified using standard fluorescent labelled ligands specific to the applied proteins (Morgan *et al* 1995, Bernard *et al* 1998, Kane *et al* 1999, Bernard *et al* 2000, Michel *et al* 2001).

In this paper, the functionality of lectins patterned with the microcontact printing technique is studied using atomic force microscopy.

Recently, atomic force microscopy (AFM) was used to measure the force of interaction in ligand–receptor pairs such as avidin–biotin and antigen–antibody pairs and complementary strands of DNA (Yuan *et al* 2000, Hinterdorfer *et al* 1996, Meiners and Quake 2000) due to its ability to measure the forces of interaction between single protein molecules with force resolution down to tens of piconewtons.

Lectins have a high affinity to specific sugar residues which has been used in different kinds of bioassays for studying the glycoprotein and carbohydrate recognition processes in mitogenic assays, characterization of malignant cells, glycoprotein purification etc (Song *et al* 2003, Hampel *et al* 1999, Mamoru *et al* 2004, Popov *et al* 2000, Stewart *et al* 1996).

The interaction force occurring in the process of mannose residue recognition by patterned lectins was studied using atomic force microscopy. The chosen molecular complex consists of lectin concanavalin A (Con A) and glycoprotein carboxypeptidase Y (CaY), since Con A recognizes mannose composed carbohydrate moieties attached to proteins.

The AFM was used to verify the biological activity of proteins patterned on a hard substrate using the microcontact printing technique. The results were compared with those obtained by protein immobilization directly from the protein solution. The images of the surface

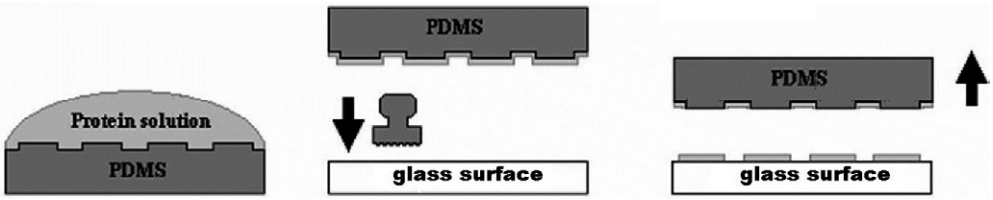

Figure 1. The idea of microcontact printing with a PDMS stamp used to translate a protein pattern onto a glass surface.

topography and the unbinding force maps correlated well with the chemical diversity of the samples investigated.

2. Materials and methods

2.1. Protein immobilization

Prior to protein immobilization, glass cover-slips were washed once in ethanol and then immersed in acetone for 5 min. They were dried and exposed to ultraviolet light (UV) for 30 min. Next, the cover-slips were incubated in 10% water solution of 3-aminopropyl-triethoxysilane (APTES, Fluka) for 1 h in order to enrich their surface in amino groups. Afterwards, they were washed once in alcohol and once in Tris buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH = 7.6, Fluka). Next, the glass surface was activated by incubation in 2.5% glutaraldehyde (Fluka, in TBS) for 30 min. Afterwards, the cover-slips were rinsed once with TBS and incubated in the 0.01 mg ml^{-1} solution of Con A (concanavalin A from *Canavalia ensiformis*, Jack Bean, Type VI, Sigma) in TBS for 1 h, and rinsed again with TBS.

In order to ensure the binding capability of the CaY–Con A complex, the TBS buffer was supplemented with 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$. All solutions were prepared using deionized water (Cobrabid water purification system, 0.08 μS). Measurements on prepared samples were made immediately.

2.2. The microcontact printing technique (μCP)

The polymeric stamps were prepared from poly-dimethylsiloxane (PDMS, Sylgard 184) as replicas from silicon moulds. They were obtained from the Polymer Group of the Solid State Physics Department, Institute of Physics of Jagiellonian University in Kraków. Two different kinds of surface patterns were used: (1) parallel lines with width of 3 μm and height of 1.5 μm, (2) periodic circles with diameter of 5 μm and depth of 1.5 μm.

The stamp surface was covered with the protein solution (0.01 mg ml^{-1} Con A in water, pH = 7.6) for 1 h. Next, the solution was removed carefully from the surface and the stamp was rinsed in pure water and dried. Before printing, the cover-slip surface was activated with glutaraldehyde as a cross-linking agent. Afterwards, the stamp was brought into contact with the premodified glass surface (as described above for protein immobilization, with one exception: all solutions were prepared using water instead of TBS buffer in order to avoid the formation of salt crystals during drying). After 15 min, the stamp was removed and the patterned cover-slip was used for AFM measurements (see figure 1).

2.3. Tip functionalization

Bare silicon nitride cantilevers (Veeco, Germany) were treated using the above-described procedure for cover-slip modification, including glutaraldehyde activation. Afterwards,

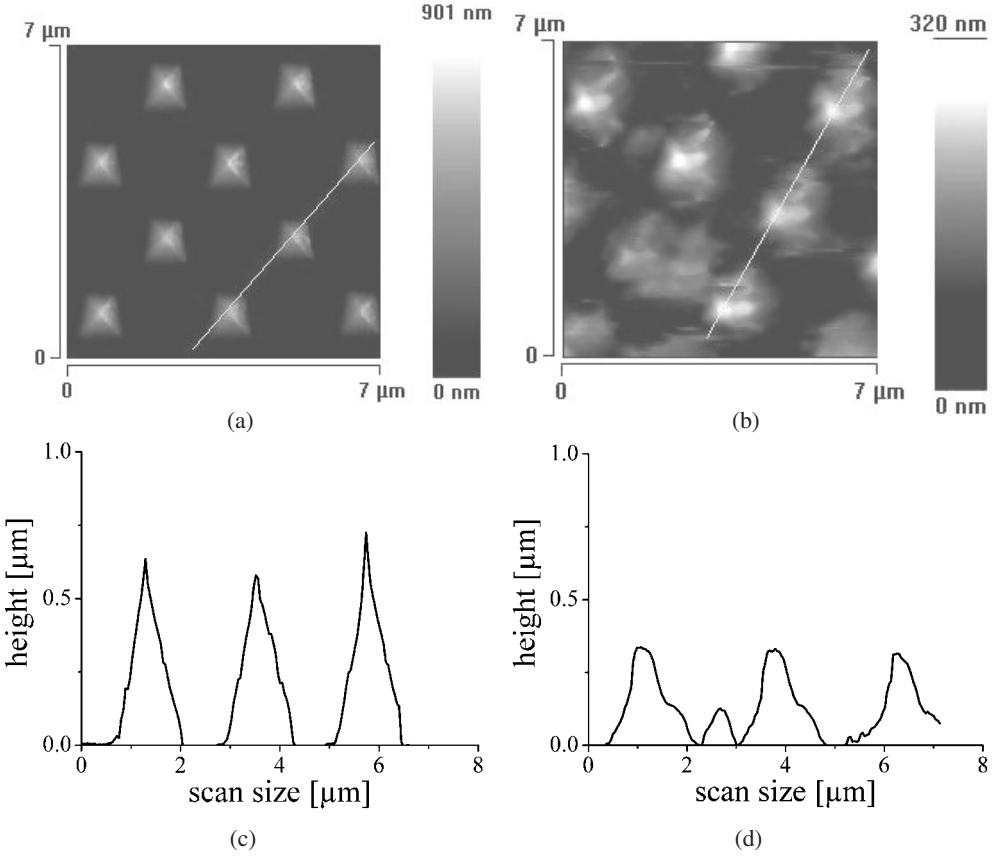

Figure 2. Images and their profile for the AFM tips: (a) before CaY functionalization (bare silicon nitride tip), (b) after functionalization with CaY, ((c) and (d)) the corresponding to AFM image profiles.

the cantilevers were rinsed in TBS buffer and incubated in 0.1 mg ml^{-1} solution of CaY (carboxypeptidase Y, Sigma) in TBS for 1 h and again rinsed in pure buffer.

After the end of each experiment, the presence of protein molecules on a tip was always verified using the TGT01 silicon standard (NT-MDT, Russia) consisting of arrays of sharpened silicon spikes. The characterization of the tips (radius of curvature and shape Bykov *et al* 1998) makes use of the convolution of the tip shape and surface morphology. Due to the scanning being that of an isolated structure that is much sharper than the probe, the resulting image is a scan of the probe itself. Figures 2(a) and (c) present such an AFM image and the corresponding profile taken with a bare silicon nitride cantilever. The average tip radius determined was 45 ± 1 nm. After protein functionalization (CaY, 0.1 mg ml^{-1} in TBS), the tip radius increased to 132 ± 9 nm (see figures 2(b) and (d)). The AFM images were performed in contact mode.

2.4. Force spectroscopy

The interaction force was measured for the CaY–Con A molecular complex. The AFM tip was modified with CaY molecules and Con A was patterned on a premodified surface. In order to compare two different methods of protein immobilization Con A was also immobilized directly from a solution.

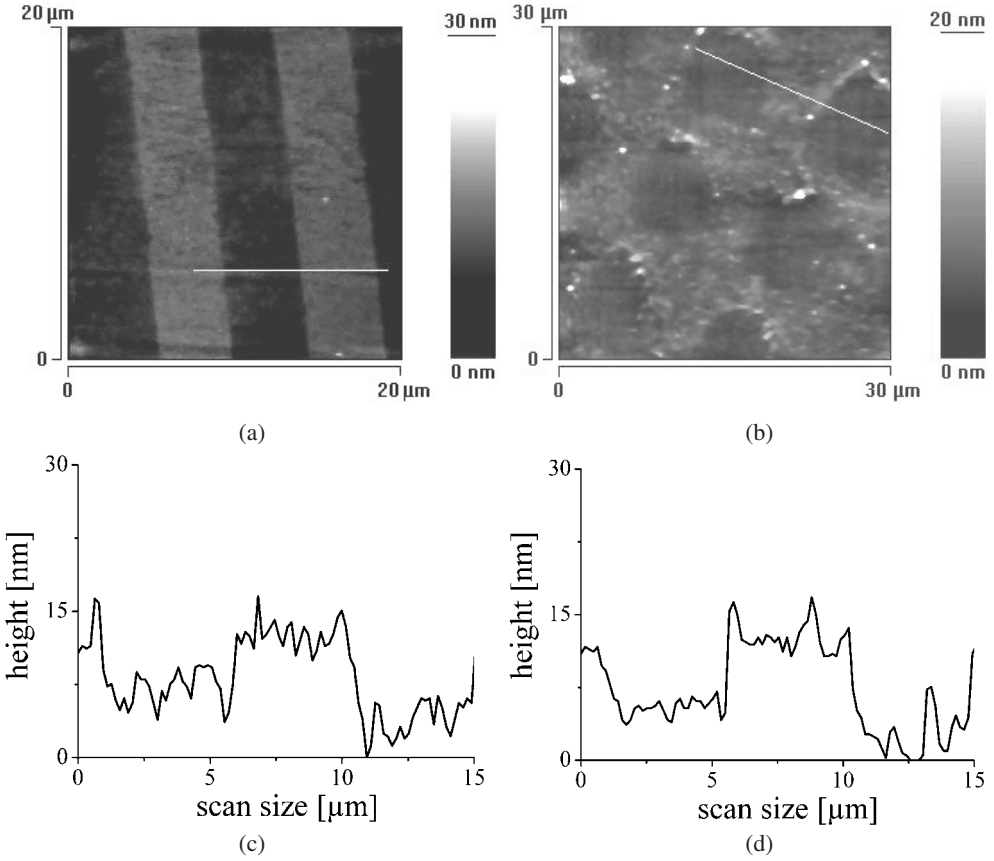

Figure 3. The AFM images of surface topography obtained by the microcontact printing technique for Con A immobilized using the PDMS stamp with two types of patterning: (a) parallel lines and (b) periodically located circles, together with the corresponding height profiles, (c) and (d).

Force measurements were carried out using a home-built AFM (Lekka *et al* 1996) equipped with a liquid cell set-up working in force spectroscopy mode. Force curves (i.e. dependences between the cantilever deflection and relative sample position) were obtained at room temperature in TBS buffer (pH = 7.6) in the presence of metal ions Mn^{2+} and Ca^{2+}. The presence of these ions is essential for the binding activity of Con A (Agrawal and Goldstein 1967). Force curves were recorded with a retraction speed of 4 μm s^{-1}. They were repeatedly collected as maps (i.e. with storage of the plane coordinates for each curve) which allowed reconstruction of the two-dimensional surface force distribution.

3. Results and discussion

3.1. Surface topography

PDMS stamping was used to print proteins on a modified glass cover-slip substrate. Both patterns (lines and circles) were imaged with bare silicon nitride tips using the AFM working in contact mode (see figures 3(a) and (b)). The corresponding profiles are presented in figures 3(d) and (e). The AFM images showed two regions—the higher one related to the protein covered

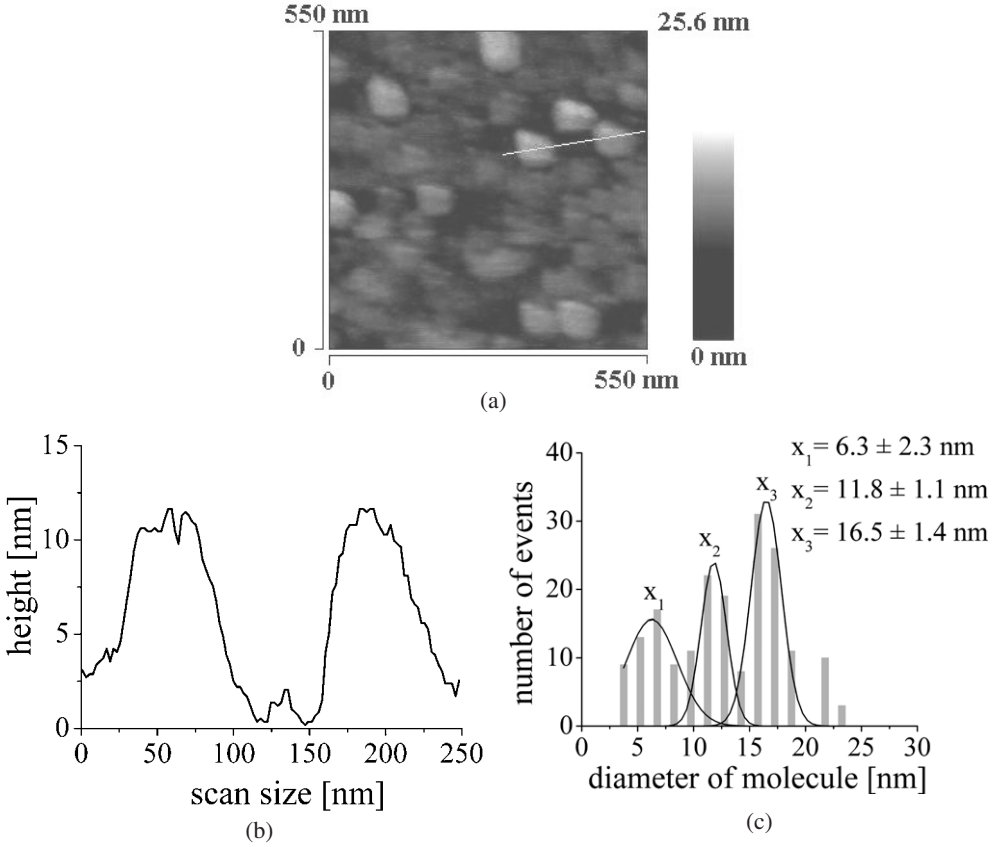

Figure 4. (a) The AFM image of the surface topography measured for Con A immobilized without any patterning method; (b) the corresponding profile and (c) the distributions of the calculated diameters for Con A molecules, determined for 200 single molecules.

surface and the lower one corresponding to the modified surface (glass cover-slips activated with glutaraldehyde). Independently of the shape of the polymeric stamp used for protein printing, the average height of the protein layer was about 15 nm. This value was compared with literature data for single Con A tetramers with dimensions 6.7 nm × 11.3 nm × 12.2 nm (Bouckaert *et al* 1996). It seems that the observed 15 nm protein layer indicates the presence of one or two layers of Con A.

The presence of proteins was also detectable using the average roughness value since the regions with Con A were rougher than the glutaraldehyde ones. The calculated average roughness values were 65 ± 20 nm for Con A and 7.2 ± 1.5 nm for glutaraldehyde surfaces, respectively, calculated from 50 areas of 1 μm^2 each.

The surface topography of the Con A immobilized on the substrate directly from the protein solution (without any patterning method) is presented in figure 4(a), where single molecules were visible. Their diameter was calculated assuming that a spherical particle of diameter D exhibits a full width at half-maximum $D' = \sqrt{D(r + 0.25D)}$ when imaged with a tip of radius of curvature r (Engel *et al* 1997). The diameter of the immobilized Con A molecules, estimated for about 200 molecules, was $r = 45$ nm (see figure 4(c)).

The histogram shows three Gaussian peaks: $x_1 = 6.3 \pm 2.3$ nm, $x_2 = 11.8 \pm 1.1$ nm, $x_3 = 16.5 \pm 1.4$ nm (errors are standard deviation values). The values obtained correlated

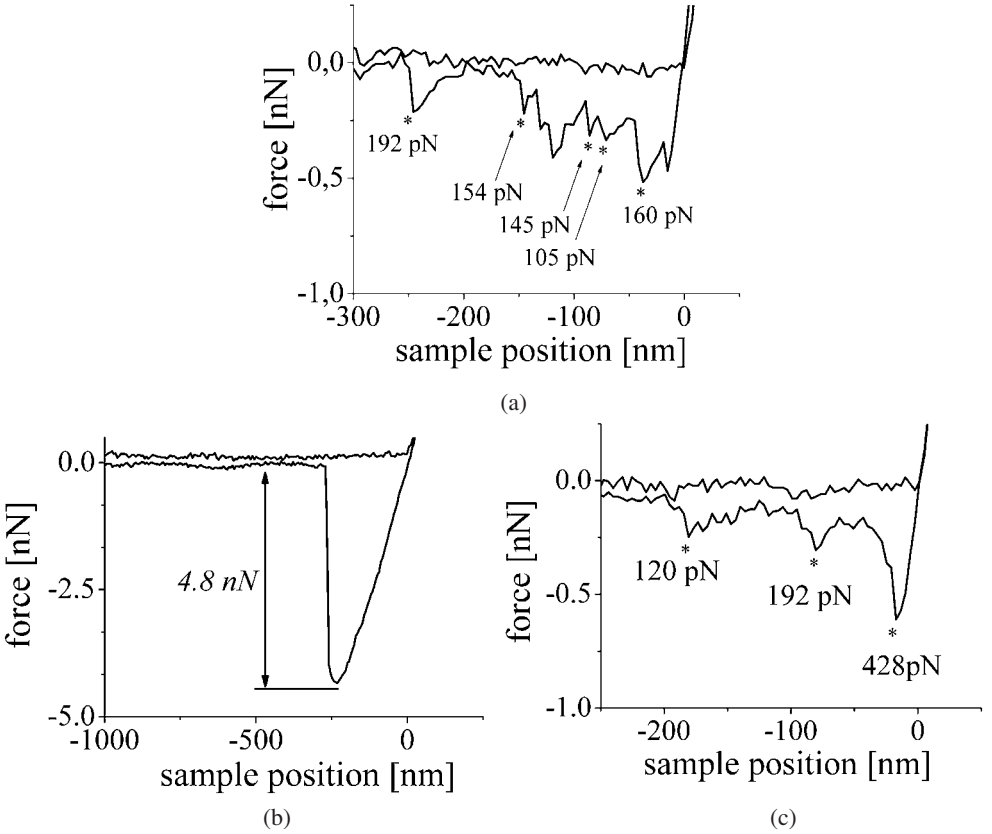

Figure 5. Typical force curves measured for the CaY functionalized tip: (a) Con A deposited on the surface using μCP; (b) glutaraldehyde activated surface; (c) Con A deposited directly from the protein solution.

quite well with the size of the single Con A tetramer (6.7 nm × 11.3 nm × 12.2 nm, Bouckaert *et al* 1996). It should be noted that in our measurements the Con A molecule contains Mn and Ca ions, while the molecule introduced by Bouckaert *et al* contains Zn and Ca ions.

According to the calculated areas under the Gaussians ($a_1 = 89.44 \pm 29.65$, $a_2 = 68.35 \pm 23.63$, $a_3 = 112.75 \pm 15.50$ respectively; figure 4(c)), the preferred molecule orientation on a surface could be 16.5 nm × 6.3 nm. The tetramer has roughly tetrahedral shape with a sugar binding site in each corner of the tetrahedron (Edelman *et al* 1972). Thus with such molecule orientation on a surface it is difficult to establish unambiguously how many molecule binding sites are exposed. At least one of them is always turned away from the surface and able to bind ligands.

3.2. Interaction force

The unbinding force between Con A–CaY molecular complexes was measured for two methods of protein deposition: directly from the protein solution and using the microcontact printing technique. The corresponding force curves obtained are presented in figures 5(a) and (c). These curves show multiple peaks which were related to several bond rupture events. Figure 5(b) presents the force curve obtained for the interaction between the CaY functionalized AFM tip and the glutaraldehyde activated glass surface.

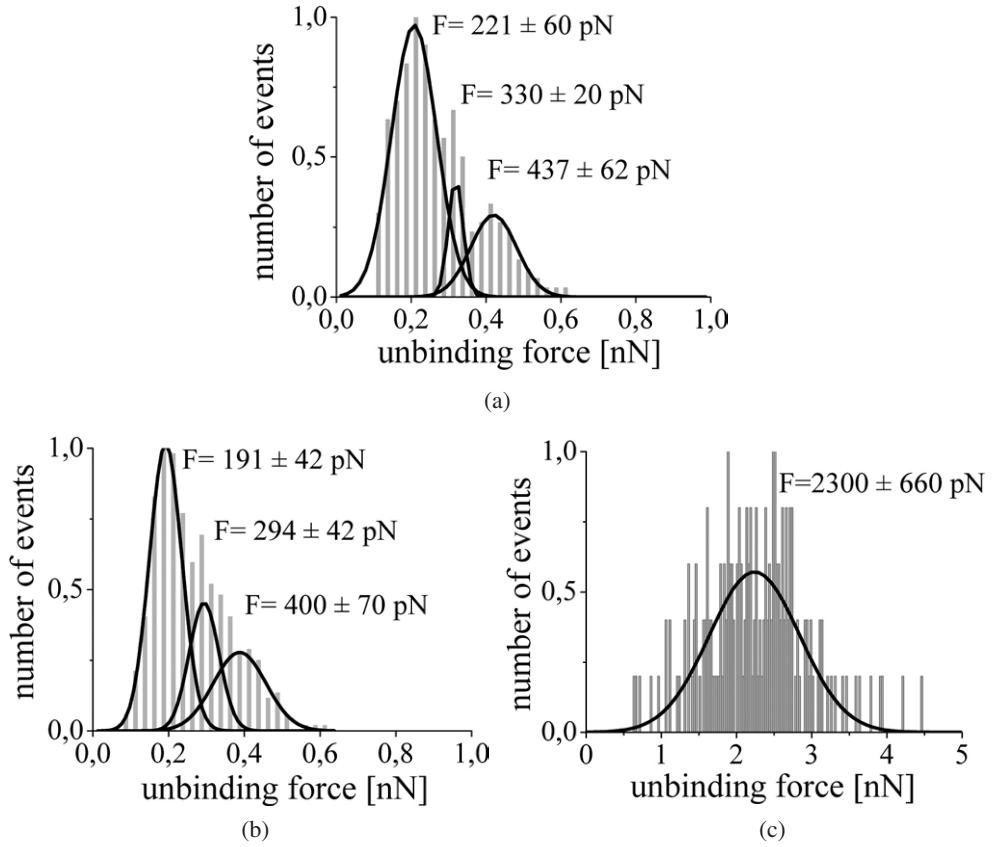

Figure 6. Force distributions for the unbinding events measured for CaY and Con A immobilized directly from the protein solution (a); Con A patterned using the microcontact printing technique (b); the force distribution obtained for a Con A functionalized AFM tip and a glass surface activated with glutaraldehyde (c). The glass cover-slip modified in the same manner as described in the materials and methods section was used as the substrate for protein immobilization.

The frequency distributions of the unbinding events measured for Con A–CaY molecular complexes are presented in the histograms (see figures 6(a) and (b)). The bin size of each histogram was set to 25 pN. The interaction force determined for the molecular complex studied never exceeded 1 nN (see figures 6(a) and (c)). However, when the CaY functionalized tip was in contact with the glutaraldehyde activated surface, the measured forces were significantly higher; the maximum distribution was above 1 nN (see figure 6(b)). This is in good agreement with expectations, since the CaY–glutaraldehyde interaction involves strong covalent binding between amino and aldehyde groups above 1 nN (Grandbois *et al* 1999).

Maxima of the force distribution were attributed to the most probable forces causing bond unbinding. The histograms were fitted with Gaussian functions from which the average values of the unbinding force were estimated for two modes of Con A deposition on the glass surface. For CaY–Con A immobilized directly from the protein solution the corresponding peaks were at 221 ± 60, 330 ± 20 and 437 ± 62 pN (see figure 6(b)). When the Con A was deposited using the microcontact printing technique, the maxima of the force distribution were at 191 ± 42, 294 ± 42 and 400 ± 70 pN. The quantized peaks observed in the histograms for the interaction force between CaY and Con A molecules indicate that a few bonds can contribute

to the measured force needed to separate two molecules from each other. The presence of more than one bond results in the appearance of peaks for multiples of the single rupture force (Chen and Moy 2000). Thus the single rupture force needed to unbind concanavalin A and carboxypeptidase Y is a difference between force values for two neighbouring peaks. In the case where Con A was immobilized on a surface without a patterning procedure the single unbinding force determined was 108 ± 2 pN. A similar value was obtained for Con A deposited onto a surface using μCP (105 ± 2 pN).

The concanavalin A belongs to a group of lectins (i.e. proteins that recognize different kinds of carbohydrate structures) that interact in a specific way with mannose residues covalently attached to another protein. The unbinding force measured by the AFM is determined by the number of interacting molecules present on both tip and substrate surfaces within the contact area. It should be taken into account that each Con A tetramer possesses four binding sites acting independently. Thus, while the AFM tip contacts the surface, a few Con A molecules react with more than one CaY molecule, forming either single or multiple bonds. Consequently, this explains the character of the retraction part of the force curves obtained for the Con A–CaY interaction, i.e. the presence of several peaks, corresponding to several bond rupture events (see figures 5(a) and (c)). Some of these peaks indicate the multivalent character of the rupture events visible also in the observed range of the unbinding force from 200 to 450 pN.

The similarity of the values of the single-bond force (108 ± 2 versus 105 ± 2 pN for the two modes of protein immobilization studied) indicates that the mode of protein deposition, in particular the application of the μCP technique, does not change the biological activity of the protein and the specific interaction remains unperturbed. These results were compared with the literature data. The measurement performed by Ratto *et al* (2004) provided the strength of the unbinding force for polymer-tethered concanavalin A and similarly tethered mannose, as 47 ± 6.9 pN. The difference between two above-mentioned measurements can be explained as follows. First, unlike our study, the experiment of Ratto *et al* was carried out to provide the interaction between single molecular complexes. Therefore, the unbinding force determined, of about 100 pN, may originate from the breaking of two single Con A–mannose bonds. Second, since the unbinding force is loading rate dependent, the increased loading rate leads to larger force values (Evans 2001). In our experiment, the loading rate was 120 nN s^{-1}, while in the experiment of Ratto *et al* it was only 10 nN s^{-1}. Also, the unbinding force obtained, of about 100 pN, could be attributed to the force needed to separate one single bond between Con A and the mannose based carbohydrate moiety of carboxypeptidase Y.

The unbinding force between Con A and mannose was also determined in other experiments, yielding values of 117 ± 41 pN (Touhami *et al* 2003b) 96 ± 55 pN (Touhami *et al* 2003a) or a range from 75 to 200 pN (Gad *et al* 1997). These values are in good agreement with our data.

3.3. Two-dimensional distributions of the unbinding events

Together with the interaction force, two-dimensional distributions of the unbinding events were determined (see figure 7). The observed pattern in the unbinding event distribution corresponded to the surface topography.

The force distribution determined from the unbinding maps showed two peaks that were correlated with two chemically different regions. The lower force values were present in the Con A covered substrate regions, while the higher forces were observed for surfaces activated only with glutaraldehyde, in the absence of Con A (see figure 8).

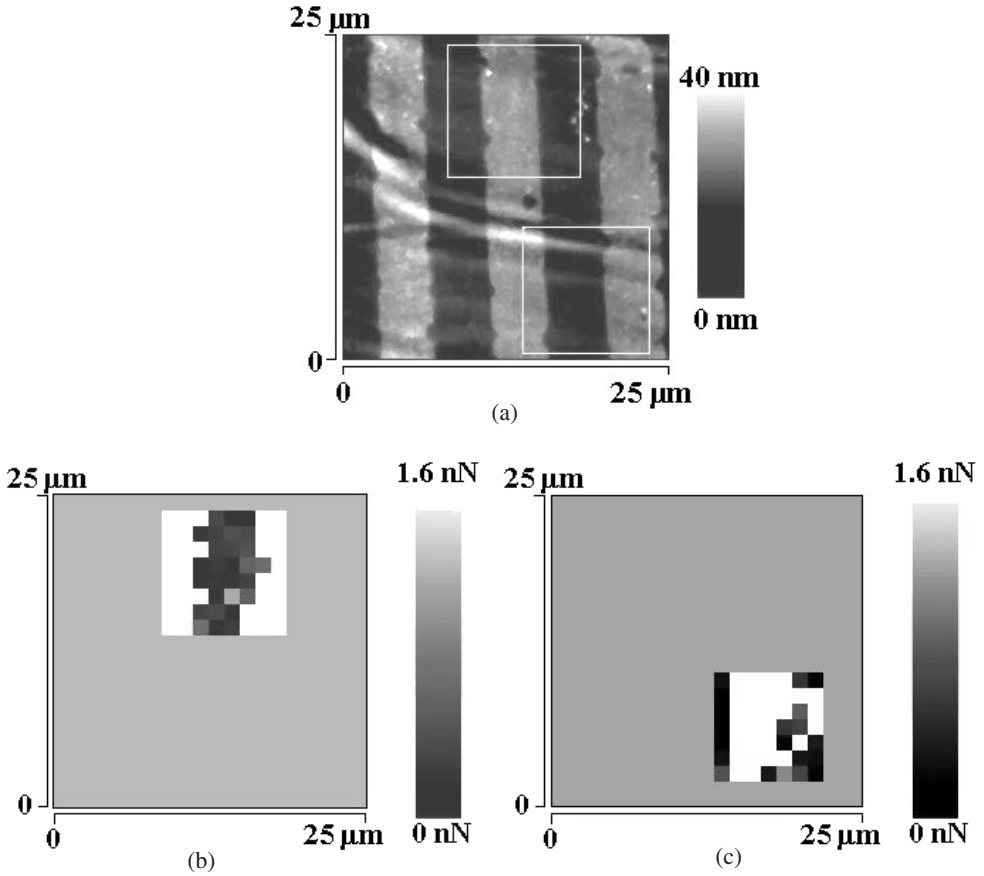

Figure 7. (a) The surface topography of the Con A substrate deposited using the μCP technique; ((b), (c)) two corresponding maps of the unbinding events from the regions marked in figure 7(a) (two squares). White corresponds to high values of the unbinding force (above 1.6 nN), grey to smaller ones (below 1 nN).

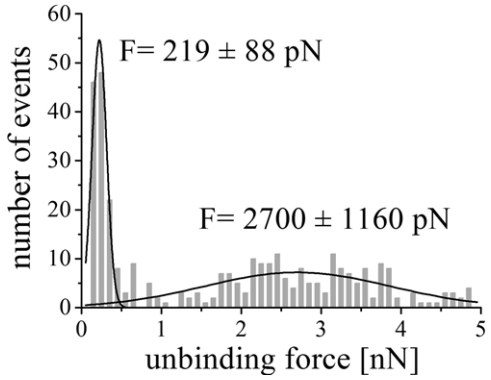

Figure 8. The histogram of the measured unbinding force between CaY and Con A determined from the map of unbinding events. Two visible maxima were fitted with Gaussian functions. The lower maximum corresponds to the unbinding force between CaY and Con A while the larger one was observed for the unbinding between CaY and glutaraldehyde activated surfaces.

4. Conclusions

Atomic force microscopy is a method becoming more and more established for determining the force of interaction between molecules, via the force spectroscopy mode.

In the present study, the AFM demonstrated its capability for analysing the quality of the deposition of a protein pattern using a PDMS stamp via measurements of the surface topography. Force spectroscopy was employed to study the molecular interaction between Con A deposited on a glass surface and a CaY functionalized AFM probe, indicating that the microcontact printing technique does not change the biological activity of the patterned protein. The unbinding force obtained was compared with that measured for Con A deposited directly from the protein solution.

Acknowledgments

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