Feeling sugar-protein interactions using carbon nanotubes : a molecular recongition force microscopy study

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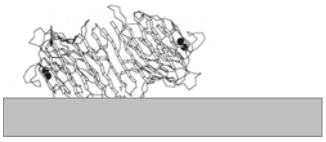
Chapter 4

Covalent immobilization of single proteins on mica for molecular recognition force microscopy

Immobilization of single proteins to flat surfaces in liquid is usually required in order to study structure and function of proteins by atomic force microscopy (AFM). On the other hand, the activity of the proteins, i.e. the specific binding capability of a receptor to its ligand, should not be blocked by the immobilization. These requirements seem to be conflicting and very often form the bottleneck for molecular recognition force microscopy (MRFM) studies.[1] Furthermore, in order to be able to detect a sufficient number of unbinding events between a ligand on the AFM tip and a receptor on the surface in MRFM, a high density of receptors on the surface is needed. We created a matrix of proteins that are covalently bound to mica with only one short (around 1 nm) tether per protein. We did this by introducing primary amine groups on the mica, coupling ethylene glycol bis-NHS esters to these and proteins to the other end of the NHS-esters via their lysin residues.[2] Proteins that are immobilized in this way are likely to be active, in spite of their firm attachment to mica. In the case of a small, globular lectin, we achieved a density of 230 dimers per μm^2 on mica. The surface roughness of mica after modification with ethanolamine and bis-NHS ester was less than 0.5 nm. This covalent immobilization technique produces a high density of individual proteins on a flat surface in liquid, with a surface roughness smaller than previously reported.



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4.1 Introduction

In order to study receptor-ligand interaction on the single-molecule scale, a special type of atomic force microscopy (AFM) technique called molecular recognition force microscopy (MRFM) can be used.[3,4,5,6,7,8] This involves detection of the interaction between the ligand bound to the scanning probe tip and receptors on a surface. MRFM experiments can be performed in liquid and at room or body temperature, which makes it possible to study the relation between structure and function of a protein under physiological conditions. A historical background of AFM in receptor ligand research was given in § 1.2.

Five requirements determine whether a surface is suitable as a substrate for MRFM. First, the surface has to be flat on a nanometer scale over a lateral range in the order of one micrometer, in order to distinguish single proteins from the roughness of the supporting surface. Suitable surfaces in this respect are for example mica, graphite, ultra-flat silicon oxide, gold and silicon nitride. Second, a strong bond should be formed between the surface and the proteins, to avoid that the probing tip moves or removes the proteins. Third, non-specific adhesion between tip and surface should be minimized. Fourth, the density of proteins on the surface should be high enough, at least 100 proteins per μm^2, to achieve a high frequency of recognition events. On the other hand, in order to distinguish individual proteins, the density should not be so high that proteins touch each other. Finally and very importantly, the ability of the protein to bind to its ligand should be preserved.

In some cases, non-specific adhesion is sufficient to immobilize the proteins on the surface, and no surface modification is necessary.[9] Particularly, proteins that carry a net positive charge at the pH conditions required for the experiments, i.e. highly basic proteins such as lysozyme or avidin, have been immobilized electrostatically on freshly cleaved, non-modified mica, which is negatively charged.[8,10,11] Negatively charged proteins or membrane fragments have been immobilized on mica using a buffer solution with bivalent cations,[12,13] or by covering mica with a thin layer of poly-L-lysine, which changes the surface into a positively charged surface.[14]

If the non-specific adhesion of the proteins is not sufficiently strong, covalent binding is necessary. This is especially true for force spectroscopy measurements, where the interaction between the protein and surface must be stronger than the forces one wishes to probe.[15] Strong binding between a protein and a gold surface can be achieved using thiols.[16] However, although free thiols can be generated,[1] not many native proteins have accessible thiol groups.

Binding proteins covalently to mica, silicon nitride or silicon oxide surfaces often involves the creation of free amine groups that

are coupled to surface silanols. One way to do this is silanization of mica[17] or silicon nitride surfaces[11] using aminopropyltriethoxysilane (APTES), which typically leads to a density of 1600 proteins per μm^2 and a roughness of 2-4 nm.[17] APTES-treated mica surfaces have been exposed recently to glutaraldehyde for the fixation of chromatin.[18] In this paper, we used esterification of mica surfaces,[5,11,17,19] because this procedure results in a low surface roughness of 0.5 nm (Figure 1). We subsequently anchored single proteins covalently to this surface via only one short tether per protein, which considerably decreases the risk of interfering with the biological function of the protein. The described procedure is thought as a general approach that can be applied to many proteins, provided that they have accessible lysine groups.

We are particularly interested in sugar binding proteins. Sugars are very important in molecular recognition; the sugar pattern on the outer membrane of a cell, for instance, makes it recognizable to other cells, through the specific binding of membrane-associated lectins. We study three different types of lectin: *Pisum sativum* (garden pea) lectin (PSL), Mannan Binding Lectin (MBL), and Wheat Germ Agglutinin (WGA). PSL plays a role in the root nodule symbiosis, in which *Rhizobium* bacteria from the soil are hosted within the root of the plant and fix atmospheric nitrogen. This enables plants to grow without added nitrate or ammonia.[20,21] MBL is a carbohydrate-binding protein present in the blood stream of mammals, as part of the innate immune system.[22] MBL recognizes, and binds to sugar patterns on microbial surfaces. Binding activates MBL-associated serine proteases and the enzymatic cascade, called "complement", thus initiating an immune response. However, the molecular mechanisms of activation remain speculative.[23] WGA is a defense plant lectin that was found to bind to carbohydrate moieties on the membrane of vesicles that are shedded from erythrocytes. These vesicles are crucial in a protection strategy of the red blood cell against the destruction by complement. The red blood cell can rapidly release vesicles containing the membrane attack complement and in this way survive the attack. The specific binding between WGA and the vesicle membrane has been used to immobilize these vesicles in liquid on mica. First, WGA had been covalently attached with a short linker to the mica surface using the esterification procedure that we have also used (see § 4.2). In this way, the sizes and structure of the vesicles were studied by AFM.[2] The fact that the immobilized WGA still binds to the sugars on the vesicle membrane shows that our immobilization procedure does not interfere with the sugar binding activity of WGA. Furthermore, whole cells have been immobilized through binding of the glycocalix to immobilized lectin. In addition to lectin, we also studied a protein that mediates cell-cell interaction, called cadherin.[24] Cadherin plays an important role in the intercellular adhesion between endothelial cells.

In § 4.2, all the experimental details of surface modification, protein immobilization and AFM imaging are given.

4.2 Experimental procedures

The surface chemistry of mica and that of silicon nitride are fairly similar. Both expose silanol groups, although it is not clear whether the silanol groups on mica are present immediately after cleaving,[25] or introduced during subsequent chemical modifications. The density of silanol groups on mica can be increased by water plasma treatment.[26]

An amine-terminated mica surface was created by spontaneous esterification between the silanol (Si-O-H) groups on freshly cleaved muscovite mica surfaces and ethanolamine. Ethanolamine was melted at 75 ° C in dry DMSO at a concentration of 0.55 g/ml. This step determines the density of amine groups that are created on mica. The competing reaction to the esterification between silanol groups and ethanolamine is the hydrolysis of the silanol groups. For this reason we use molecular sieve beads to extract all the water from the ethanolamine-DMSO solution.

The primary amines that are exposed on the mica surface after this first modification step can be used for the binding of another functional group. We used a bis-succinimide ester (bis-NHS ester) that forms an amide bond with the primary amines. A sketch of this reaction is shown in Figure 2. For this second step, it is important to avoid the formation of loops of the bis-NHS ester, because one of the two succinimides is required for subsequent protein binding. However, this is not a problem because the mean distance between surface amine groups is larger than the length of the bis-NHS spacer. In addition, we use a high concentration of bis-NHS, which will quickly saturate the amines on the surface. For a fast reaction rate of NHS to amines and to avoid hydrolysis of the NHS-residues, we use very basic and dry conditions (chloroform with tri-ethylamine).

With these two modification steps, we have created an NHS-terminated surface. This surface is now ready to be used for binding proteins in a covalent way. The free NHS-residue of the surface-bound bis-succinimide can form an amide bond to a lysine group of a protein. The estimated length of the bis-succinimide ester is around 1 nm. The surface density of primary amines on silicon nitride after the first step in the modification process was determined to be 723 groups per μm^2, using a marker enzyme assay with horse radish peroxidase.[17] In our AFM studies (Figure 3), we find a density of 230 PSL dimers per μm^2, from which we conclude that a dimer is linked through 1 or 2 of its lysine groups via the bis-succinimide linker to an amine group on the mica surface. The density of coupled proteins is fairly homogeneous over the whole mica surface.

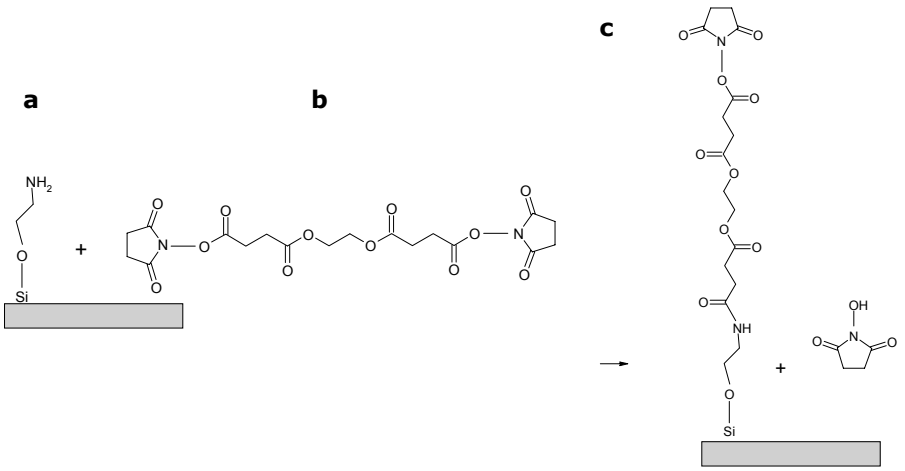

Figure 1 Sketch of the binding reaction of a bis-NHS (b) ester to amine-modified mica (a). A protein can now be bound to the free end of the NHS-ester (c).

Surface modification

As first step in the modification process, a solution of ethanolamine from Sigma Chemicals in dry dimethylsulfoxide, DMSO (6.6 g in 12 ml) was prepared and heated up to 70 °C. To keep the solution water-free, molecular sieve beads (0.3 nm) were added. Freshly cleaved Muscovite mica sheets were incubated overnight in this solution. The mica sheets were rinsed 4 times in DMSO (75 °C) and twice in ethanol (room temperature). The mica sheets were then dried in a nitrogen flow. At this point, the amine-modified mica was used immediately for the next modification step, or stored in a desiccator for up to several weeks (for a detailed description see Reference 17).

The second step was binding of a bis-succinimide ester to the primary amines now present on the mica. The amine-modified mica was incubated 3 h in a solution of ethylene glycol-bis-(succinimidyl-succinate) from Sigma Chemicals in chloroform (1 mg/ml) with triethylamine (0.5 % v/v). The mica sheets were washed in chloroform and dried in a nitrogen flow. The mica sheets were immediately used for protein coupling.[2]

Protein immobilization

Further sample preparation for the experiments on PSL consisted of the following steps: 75 µl of PSL of 1 µg/ml in Tris (0.1 M, pH 7.2) was first centrifuged for 5 min at 13000 rpm and

incubated for 3 h on mica modified using reaction 1 and 2 as described above. After incubation the sample was rinsed with Tris.

MBL samples were prepared in the following way: 50 µl of a recombinant MBL solution of 3 µg/ml in TBS (0.1 M, pH 7.4) was incubated on modified mica for 3 h.

Cadherin constructs were made by coupling two extracellular parts of cadherin monomers to one Fc fragment of IgG.[24] The cadherin construct concentration was around 1 µg/ml in an 150 mM NaCl/ 5mM $CaCl_2$/ 5 mM Hepes buffer. Before incubation the cadherin solution was sonicated for 1 min and centrifuged for 20 min at 14000 rpm. 300 µl of the cadherin solution was incubated on modified mica for 15-20 min and the surface was rinsed with NaCl/$CaCl_2$/Hepes buffer. Antibodies against cadherin were linked to the AFM tip via PEG-spacers.

Red blood cells were freshly extracted and diluted in phosphate buffered saline by 1:60 before a 70 µl droplet of these cells was placed on WGA-mica for 20 min and washed with the same buffer. Cells were then fixed in 1 % glutaraldehyde for 1 min and imaged in a fluid cell. Binding of WGA to mica was done as described above and elsewhere.[2]

AFM imaging

The AFM images shown in Figure 2 - 4 and in Figure 6 were taken in tapping mode with a Nanoscope III AFM equipped with a commercial fluid cell from Veeco. The feedback loop was driven at 5-10 % (Figure 2 - 4) or 25-35 % (Figure 6) amplitude reduction. The AFM image shown in Figure 5 was taken with a Mac mode PicoSPM magnetically driven dynamic-force microscope from Molecular Imaging, using about 10 % amplitude reduction.

PSL was imaged in Tris with a biolever from Olympus at a resonance frequency in liquid of 9 kHz and a spring constant of 0.03 N/m. The tip scanning speed was 2 µm/s.

For imaging MBL, a force modulation probe from Scientec with a resonance frequency of 30 kHz in liquid and a spring constant of 2 N/m was used. The tip speed was 10 µm/s.

The Cadherin image was made in MAC mode using a cantilever from Park Scientific with resonance frequency 9 kHz and spring constant 0.1 N/m. The scan speed was 4 µm/s. The force distance curve was taken with 100 nm z amplitude and 1.2 s per curve.

4.3 Results and Discussion

Figure 2 shows a topography image of mica modified as

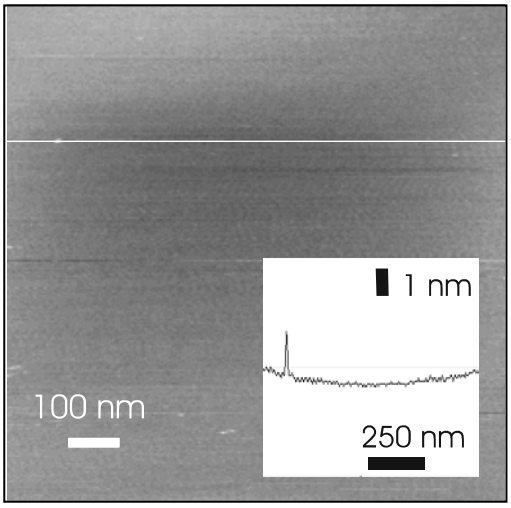

Figure 2 Modified mica surface imaged in Tris buffer in tapping mode. Image size is 1 μm^2, height is from 0 (dark) to 3 (bright) nm. The inset is a line profile taken at the position of the white line.

described above, imaged in Tris buffer (0.1 M, pH 7.2). The cross section in the inset reveals that the roughness of the modified mica determined by AFM in liquid is less than 0.5 nm. This image serves as a control of contaminants on the surface that could be caused by adsorption from possible components of the buffer. The surface appears to be apt for resolving and imaging single molecules.

The topography image in Figure 3 shows PSL covalently bound to the modified mica, imaged in liquid. On this area of 1 μm^2, 230 single structures of PSL can be resolved. Comparing the measured dimensions with the dimensions of the known crystal structure (dimer length 8 nm, dimer width 5 nm), we assume that the smallest of these structures are dimers. The dimeric form is also known to be the most stable form of PSL. While most of the imaged lectins on the surface appear in the dimeric form, several clusters of two or more dimers are visible. Thus, the stable and dense attachment of defined structures is ideal for single molecule recognition force microscopy. To check if the binding of PSL to the modified mica was indeed covalent, PSL was absorbed to untreated mica, which did not result in any binding of PSL to the surface.

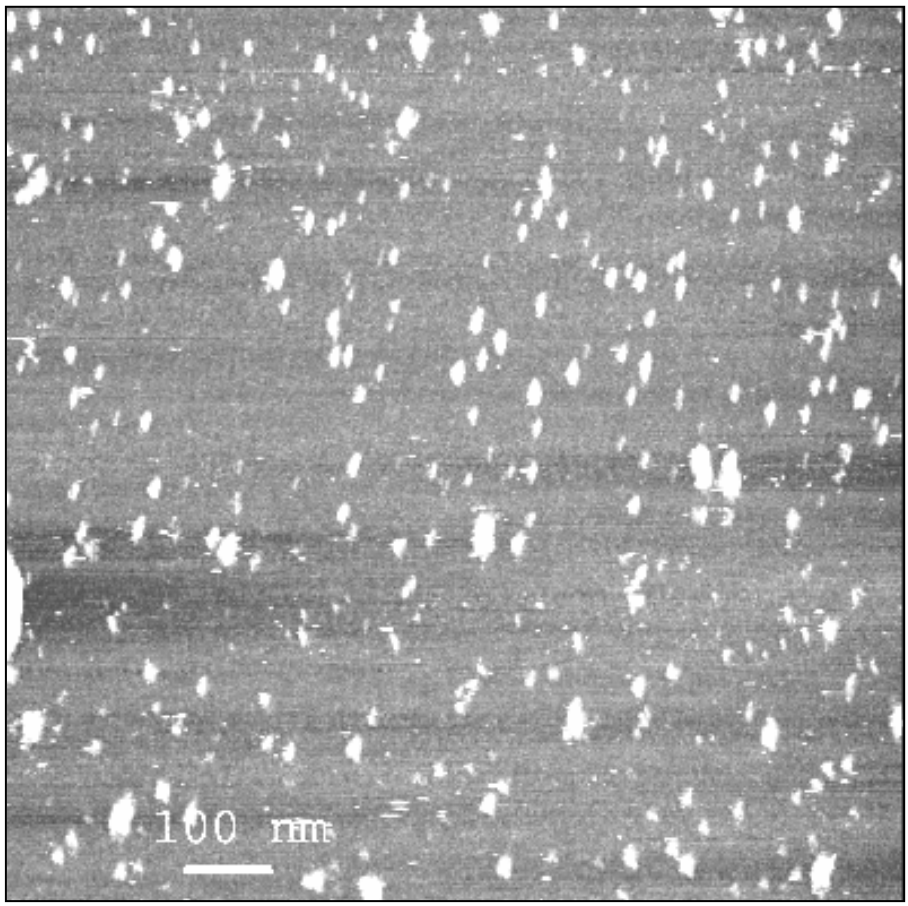

Figure 3 Pea lectin immobilized on a modified mica surface, imaged in Tris buffer in tapping mode. Image size is 1 μm^2, height is from 0 to 8 nm. The surface coverage is 230 dimers and clusters.

Recombinant MBL on modified mica imaged in liquid is shown in Figure 4. MBL consists of 2-6 subunits, each made up of 3 identical polypeptide chains. These chains each contain a tail region, a collagen-like domain where the three chains are tightly twisted around each other, an alpha helical neck region and finally terminate in globular, carbohydrate recognizing domains, one for each chain. The subunits are connected through the cystein-rich tail region. The final MBL thus has a sertiform (sertula = small umbel) configuration with a hub or short stem from which the subunits branch out as flowers.

This characteristic shape is seen in Figure 4, and on some of the molecules the hub shows up as a tall (white) spot in the

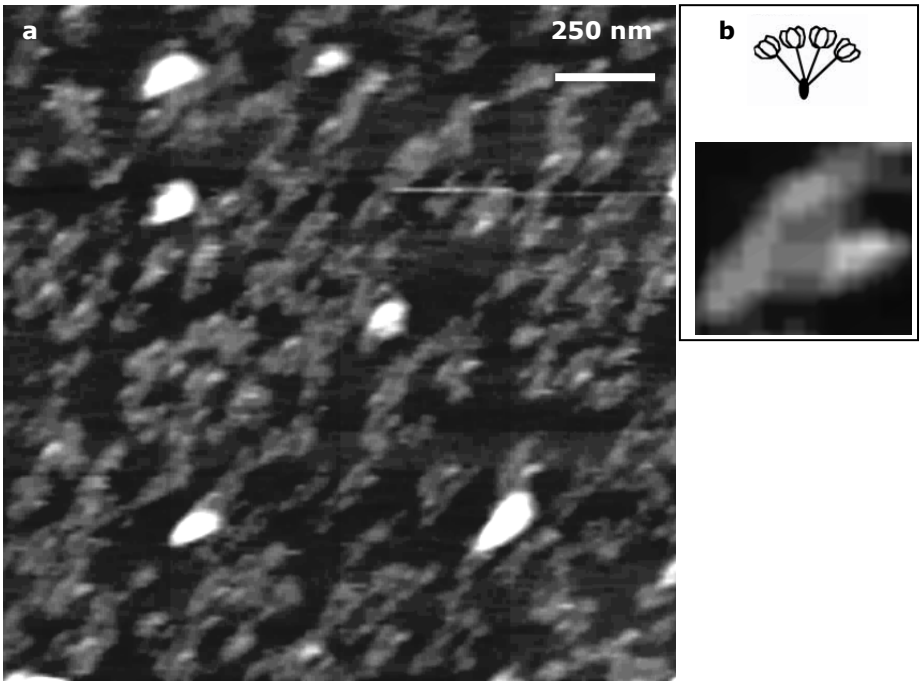

Figure 4 (a) Recombinant mannan binding lectin immobilized on modified mica, imaged in TBS buffer in tapping mode. Image size is 1.6 μm x 1.6 μm, height from 0 to 5 nm. Presented is a software zoom of a 5 μm x 5 μm image. (b) Sketch[22] of an rMBL consisting of 4 subunits, and a zoom from the AFM image (100 nm x 100 nm); approximately to scale. The enlargement of the features in the AFM picture is due to a combination of drift, tip convolution, and partial mobility of the protein on the surface. Image was made by Henriette Jensenius.

molecule. It is thus seen that the immobilization procedure developed here allows for sub-molecular resolution.

Cadherin constructs [24] were covalently bound to modified mica, see Figure 5. Single cadherin constructs as well as clusters formed in the calcium-containing buffer were observed. In order to perform molecular recognition experiments, an antibody against cadherin was coupled to the AFM tip for studying the interaction force to cadherin immobilized on mica in liquid. The force distance curve in the inset shows that the antibody on the AFM tip binds to the immobilized cadherin. From this, we conclude that the immobilization process does not obscure the specific binding capability of the cadherins to the antibody. The specificity was further supported by a blocking experiment. The cadherin molecules on the surface were bound with antibodies and as a consequence no specific interaction between tip and surface was observed.

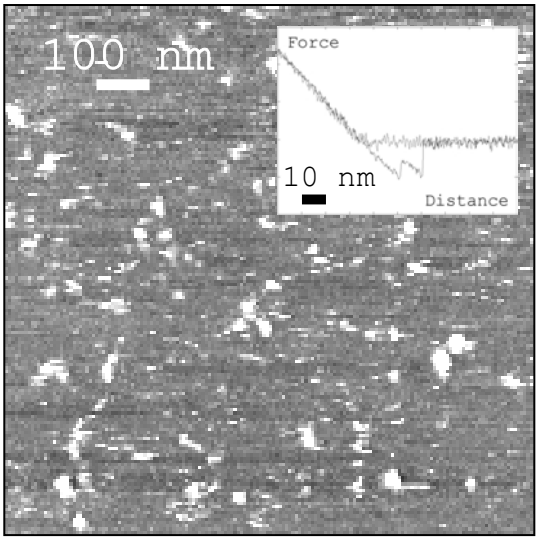

Figure 5 Cadherin immobilized on modified mica, imaged in $NaCl/CaCl_2$/Hepes buffer in MAC-mode. Image size is $1\mu m^2$, height from 0 to 0.9 nm. The inset is a force distance curve taken on this surface with an AFM tip that was functionalized with an antibody against cadherin. The force distance curve shows unbinding between cadherin and an antibody. The image was made by Maarten van Es.

WGA covalently bound to mica via bis-NHS ester was used as a functional matrix to attach live red blood cells via their carbohydrate moieties in the membrane to the surface for high resolution imaging (Figure 6).

The erythrocytes, showing expected dimensions about 5 μm in height and 10 μm in diameter, as can be seen in Figure 6 a, remained stably bound to the matrix during the imaging procedure, indicating that the fixation of each single erythrocyte occurred over many WGA/carbohydrate bonds, due to the high surface densities of both the WGA molecules on the surface and the sugars in the cell membrane, respectively. This stable attachment even allowed to resolve fine structural details on the membrane surface (Figure 6 b). Since almost every cell contains extracellular carbohydrate moieties, this attachment protocol may serve as general strategy to immobilize cells for AFM experiments.

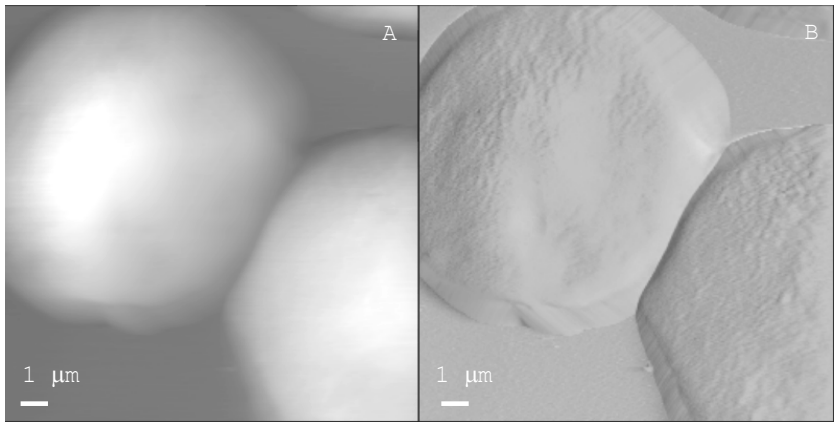

Figure 6 Tapping mode topography (a) and amplitude (b) image of a red blood cell specifically bound to a WGA matrix on mica. Image size is 10 μm with heights ranging from 0 to 3.3 μm. The image was made by A. Kamruzzahan and C.M. Stroh.

4.4 Conclusions

We have developed a method to prepare a high density of homogeneously distributed, active, single proteins covalently bound to mica with only one short tether. This was achieved by introduction of primary amine groups on mica and coupling one NHS-end of ethylene glycol bis-NHS esters to these amines. The free NHS-end of the surface-bound bis-succinimide ester then formed an amide bond with a lysine group of a protein.

All five requirements itemized in the introduction are fulfilled by the mica surfaces modified by the presented method: (i) The surface roughness is less than 0.5 nm. (ii) A covalent bond is formed between the proteins and the mica surface. (iii) Our procedure does not leave a sticky layer on the whole mica surface, it just anchors the proteins to the mica very locally. This avoids the introduction of a sticky layer that would cause non-specific adhesion between tip and surface. (iv) The maximum density of the proteins on mica that we achieved was 230 dimers per μm^2 for PSL. The matrix of active receptors obtained in this way is perfectly suited for single molecule recognition studies: The chance of detecting an unbinding event when scanning this matrix with a specific ligand coupled to the AFM tip is high, while still single proteins are addressed. (v) The preservation of the binding activity of the receptors after covalent immobilization onto mica was confirmed by single molecule antibody/cadherin recognition events.

[1] P. Hinterdorfer in Methods in cell biology, Vol. **68** (Eds.: B.P. Jena, H. Hörber), Elsevier Science, San Diego, 2002, pp.115-137
[2] U. Salzer, P. Hinterdorfer, U. Hunger, C. Borken, R. Prohaska, Blood 2002, **99**, 2569-2577
[3] G.U. Lee, D.A. Kidwell, R.J. Colton, Langmuir 1994, **10**, 354-357
[4] E.L. Florin, V.T. Moy, H.E. Gaub, Science 1994, **264**, 415-417
[5] P. Hinterdorfer, W. Baumgartner, H.J. Gruber, K. Schilcher, H. Schindler, Proc. Natl. Acad. Sci. USA 1996, **93**, 3477-3481
[6] M. Grandbois, W. Dettmann, M. Benoit, H.E. Gaub, J. Histochem. Cytochem. 2000, **48**, 719-724
[7] O.H. Willemsen, M.M.E. Snel, K.O. Van der Werf, B.G. De Grooth, J. Greve, P. Hinterdorfer, H.J. Gruber, H. Schindler, Y. Van Kooyk, C.G. Figdor, Biophys. J. 1998, **75**, 2220-2228
[8] A. Raab, W. Han, D Badt, S.J. Smith-Gill, S.M. Lindsay, H. Schindler, P. Hinterdorfer, Nature Biotechn. 1999, **17**, 902-905
[9] E. Friis, PhD thesis, Technical University of Denmark, 1999
[10] M. Radmacher, M. Fritz, H.G. Hansma, P.K. Hansma, Science 1994, **265**, 1577-1579
[11] K.R. Riener, C.M. Stroh, A. Ebner, C. Klampfl, A.A. Gall, C. Romanin, Y.L. Lyubchenko, P. Hinterdorfer, H.J. Gruber, Analytica Chimica Acta 2003, **479**, 59-75
[12] G. Kada, L. Blayney, A. Raab, S. Fleischer, H. Schindler, F. Anthony Lai, P. Hinterdorfer, Single Mol. 2000, **1**, 174
[13] A. Stamouli, S. Kafi, D.C.G. Klein, T.H. Oosterkamp, J.W.M. Frenken, R.J. Cogdell, Th.J. Aartsma, Biophys. J. 2003, **84**, 2483-2491
[14] D.J. Müller, A. Engel, M. Amrein, Biosensors & Biolectronics 1997, **8**, 867-877
[15] P. Hinterdorfer, H.J. Gruber, F. Kienberger, G. Kada, C. Riener, C. Borken, H. Schindler, Colloids and Surfaces B: Biointerfaces 2002, **23**, 115-123
[16] M. Grandbois, M. Beyer, M. Rief, H. Clausen-Schaumann, H. E. Gaub, Science 1999, **283**, 1727-1730
[17] P. Hinterdorfer, K. Schilcher, W. Baumgartner, H.J. Gruber, H. Schindler, Nanobiology 1998, **4**, 177-188
[18] H. Wang, R. Bash, J.G. Yodh, G.L. Hager, D. Lohr, S.M. Lindsay, Biophys. J. 2002, **83**, 3619-3625
[19] M. Trau, B.S. Murray, K. Grant, F. Grieser, J. Colloid Interface Sci. 1991, 148-1, 182-189
[20] C.L. Díaz, L.S. Melchers, P.J.J. Hooykaas, B.J.J. Lugtenberg, J.W. Kijne, Nature 1989, **338**, 579-581
[21] S. Stracke, C. Kistner, S. Yoshida, L. Mulder, S. Sato, T. Kaneko, S. Tabata, N. Sandai, J. Stougaard, K. Szczyglowski, M. Parniske, Nature 2002, **417**, 959-962
[22] M.W. Turner, R.M.J. Hamvas, Rev. Immunogen. 2000, **2**, 305-322
[23] U. Holmskov, S. Thiel and J.C. Jensenius, Annu. Rev. Immunol. 2003, **21**, 547-578
[24] W. Baumgartner, P. Hinterdorfer, W. Ness, A. Raab, D. Vestweber, H. Schindler, D. Drenckhahn, Proc. Natl. Acad. Sci. USA 2000, **97**, 4005-4010
[25] J. Peanasky, H.M. Schneider, S. Granick, Langmuir 1995, **11**, 953-962
[26] E. Kiss, C.-G. Gölander, Colloids and Surfaces 1990, **49**, 335-342