Imaging streptavidin 2D-crystals on biotinylated lipid monolayers at high resolution with the atomic force microscope

SIMON SCHEURING, DANIEL J. MÜLLER, PHILIPPE RINGLER, J. BERNARD HEYMANN, ANDREAS ENGEL*

M. E. Müller Institute for Microscopic Structural Biology at the Biozentrum, University of Basel, Klingelbergstr. 70, Basel CH-4056, Switzerland
Tel.: +41 61 267 22 61
Fax: +41 61 267 22 59

* To whom correspondence should be addressed:
e-mail: aengel@ubaclu.unibas.ch

abbreviated running title:
AFM on streptavidin 2D-crystals

key words: AFM, streptavidin, 2D crystal, lipid monolayer, HOPG, preparation method

SUMMARY

Streptavidin crystals were grown on biotinylated lipid monolayers at an air/water interface and transferred onto highly oriented pyrolytic graphite (HOPG). These arrays could be imaged to a resolution below 1 nm with the atomic force microscope. The surface topographs obtained were compared with negative stain electron microscopy images and the atomic model determined by X-ray crystallography. The streptavidin tetramer (60 kD) exposes two free biotin binding sites to the buffer solution, while two are occupied by the linkage to the lipid monolayer. Therefore the streptavidin 2D crystals can be used as nanoscale matrices for binding biotinylated compounds. Furthermore, this HOPG-based preparation method provides a general novel approach to study the structure of protein arrays assembled on lipid monolayers with the AFM.

INTRODUCTION

The atomic force microscope (AFM) (Binnig *et al.*, 1986) has become a powerful tool in structural biology, because topographs of biomolecules can be acquired under physiological conditions at subnanometer resolution. High resolution AFM requires atomically flat surfaces (Müller *et al.*, 1995). Frequently used supports to immobilize biological objects for imaging with the AFM are mica having a polar surface (Müller *et al.*, 1997) and functionalized gold (Wagner *et al.*, 1996) or silanized glass (Karrasch *et al.*, 1993) allowing covalent crosslinking to be achieved. These supports are suitable for the adsorption of membrane proteins reconstituted into lipid bilayers (Jap *et al.*, 1992) and of single particles such as soluble proteins (Mou *et al.*, 1996).

In this report atomically flat HOPG (highly oriented pyrolytic graphite) was used as a hydrophobic support to acquire high resolution topographs of streptavidin 2D crystals on biotinylated lipid monolayers using the AFM. HOPG is produced by deposition of carbon at high temperature (up to 3000°C) and under pressure from the gas phase (Moore, 1973). The material thus obtained consists of crystallites that are well oriented perpendicular to the basal graphite planes (Ohler *et al.*, 1997), and can easily be cleaved with scotch tape. On a macroscale HOPG is not flat, but the surface is separated into atomically flat terraces. The flatness of these plateaus assures that the surface features are specimen specific and not due to irregularities of the substrate itself. The graphite, consisting of hexagonally ordered carbon atoms, presents a nonpolar strongly hydrophobic surface.

Streptavidin is a tetrameric protein with four biotin binding sites (Green, 1975). The ease with which streptavidin crystallizes in 2D arrays on a biotinylated lipid monolayer (Darst *et al.*, 1991; Avila-Sakar & Chiu, 1996) makes it an ideal model system to investigate two-dimensional crystals grown on lipid monolayers that are transferred to HOPG. In addition, a comparison can be made with the transmission electron microscope (TEM) of the same crystals as well as the available atomic structure from X-ray crystallography (Avila-Sakar & Chiu, 1996; Hendrickson *et al.*, 1989). Topographs recorded with the AFM compare favourably with negatively stained samples and the atomic structure of streptavidin.

MATERIALS AND METHODS

Materials:

Streptavidin and biotin-LC-DPPE (biotin-longchain-dipalmitoyl phosphatidyl ethanolamine) were obtained from Pierce Ltd. (Rockford, USA), DOPC (dioleoyl phosphatidyl choline) from Avanti Polar Lipids (Birmingham, AL), mica from Mica New York (Varick Street, N. Y. 10013), HOPG from Advanced Ceramics Corporation (Cleveland, USA), and Araldit from Ciba-Ceigy (Basel, Switzerland).

Hydrophobicity measurement:

A 10 µl drop of millipore filtered H_2O was deposited on the substrates mica (Bailey, 1984), washed glass (Karrasch *et al.*, 1993), untreated glass, HOPG (Moore, 1973), and teflon. Right after deposition of the drops the diameters were photographed at 6x magnification using a binocular microscope with a mounted camera, and the diameters measured.

Crystallization of streptavidin on biotin-lipid monolayer:

Highly ordered streptavidin arrays were produced by depositing 15 µl of streptavidin solution (10 mM Tris-HCl, pH 7.5, 150 NaCl) at a concentration of 0.1 mg/ml in a Teflon well 0.5 mm deep and 4 mm in diameter (Fig. 1a). A 0.5 µl drop of the lipid mixture (0.5 mg/ml Biotin-LC-DPPE : DOPC, 1:4 (mol : mol), in chloroform : hexane, 1:1 (vol : vol)) was then deposited on top of the protein solution with a Hamilton syringe (Fig. 1a). Incubation overnight at room temperature allowed the adsorption of protein to the lipid monolayer and subsequent growing of 2D-crystals (Fig. 1b, c).

Atomic force microscopy (AFM):

Highly ordered pyrolytic graphite (HOPG) with dimensions of 3 mm x 3 mm x 1 mm was glued with water-insoluble Araldit epoxy onto a teflon disc (diameter: 11 mm). The teflon disc was glued to a steel disc (diameter: 10 mm) which was mounted in the AFM (Fig. 1d). Subsequently, the HOPG was cleaved with scotch tape, ensuring that the surfaces separate over the whole area. The surface was scanned in buffer solution (10 mM Tris-HCl, pH 7.5, 150 mM KCl) with a Nanoscope III AFM (Digital instruments, Santa Barbara, Calif.) equipped with a 120 µm scanner (J-scanner). A 120 µm long cantilever from Digital instruments (k = 0.38 N/m) was used. For recording HOPG topographs the AFM was operated at minimal force (<0.2 nN) and 4 Hz scan speed.

To adsorb 2D streptavidin crystals, the freshly cleaved HOPG was brought into contact with the monolayer on the surface of the drop in the teflon well (Fig. 1). The sample was kept wet throughout the preparation procedure. A drop of 30 µl scanning buffer (10 mM Tris-HCl, pH 7.75, 200 mM KCl) was immediately added and the specimen was mounted in the atomic force microscope (AFM). The 120 µm scanner (J-scanner) was used together with oxide sharpened Si_3N_4 cantilevers from Digital instruments with a length of 200 µm (k = 0.06 N/m). For imaging the AFM was operated at constant force mode applying minimal forces (<0.2 nN) at a scanning speed of 4-6 Hz. The images were correlation averaged with the SEMPER image processing system (Saxton *et al.*, 1979).

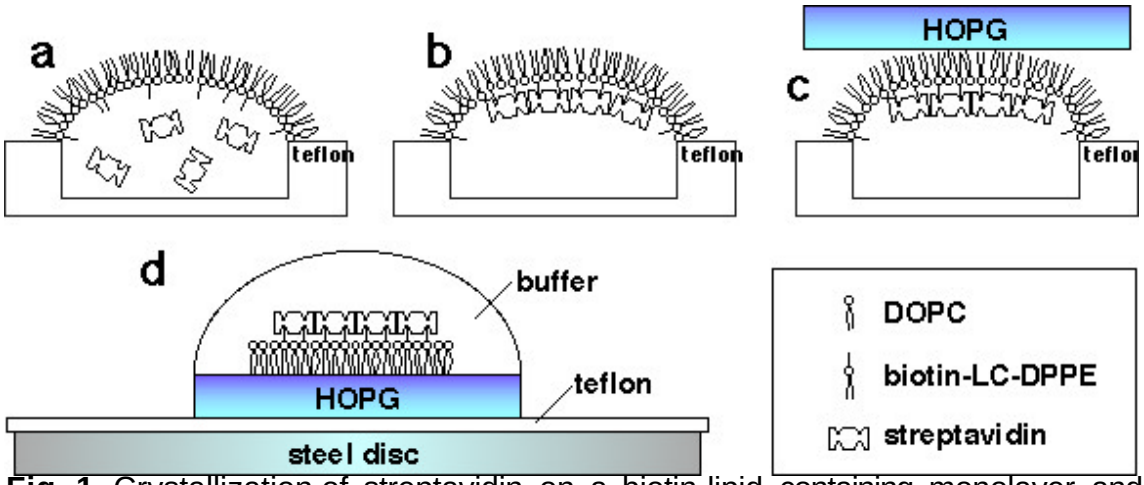

Fig. 1. Crystallization of streptavidin on a biotin-lipid containing monolayer and adsorption to HOPG: a) A 15 µl drop of streptavidin solution (0.1 mg/ml in 10 mM Tris-HCl, pH 7.5, 150 NaCl) was deposited into a teflon well and a 0.5 µl drop of lipid mixture (Biotin-LC-DPPE : DOPC, 1 : 4) was spread on the drop to form a monolayer. b) Overnight incubation at room temperature allowed streptavidin binding to the lipid monolayer and the formation of 2D-streptavidin crystals. c) The monolayer was adsorbed to freshly cleaved HOPG and d) mounted in the AFM in a drop of scanning buffer (10 mM Tris-HCl, pH 7.5, 150 KCl)

Transmission electron microscopy (TEM):

For transmission electron microscopy, samples were prepared on a copper grid covered by a parlodion film and a carbon layer. A 10 day old hydrophobic grid was deposited on the lipid monolayer covering the protein solution in a teflon well for 1 min. The grid was removed and blotted with filter paper, and subsequently washed three times with double distilled water. The specimen was negatively stained twice for 15 sec with 0.75% uranyl formate, blotted, and dried in an air stream. Micrographs were taken in a Hitachi H7000 TEM at low dose conditions at 50000 x magnification. The negatives were digitized with a Leafscan-45 (Leaf Systems Inc., Cupertino, CA) at a stepsize of 20 µm (~0.4 nm at the specimen) and selected areas were correlation averaged (Saxton & Baumeister, 1982).

RESULTS

Hydrophobicity and topography of HOPG:

The hydrophobicity of HOPG was compared with other surfaces using the sitting drop diameter method (Karrasch *et al.*, 1993). In comparison with the well-used substrates, mica and glass, HOPG exhibits an increased hydrophobicity (Table 1). As indicated by the difference in drop diameter, the difference in hydrophobicity between HOPG and silanized glass is significant. Indeed, streptavidin crystals on a biotinylated monolayer could not be successfully adsorbed to the silanized coverslip, whereas they could be transferred to HOPG reproducibly.

Table 1: Determination of the hydrophobicity of various substrates by measuring the diameter of 10 µl nanopure water droplets deposited on each substrate.

Substrate	Diameter (mm)
mica †	12.3 ± 1.9 (n=41)
glass (etched)*	7.5 ± 0.7 (n=50)
glass (washed)†	7.4 ± 1.3 (n=66)
glass (washed)*	7.4 ± 0.7 (n=50)
glass (untreated)†	4.9 ± 0.1 (n=60)
glass (silanized)*	4.1 ± 0.2 (n=40)
HOPG †	3.7 ± 0.2 (n=54)
teflon †	3.1 ± 0.1 (n=70)
spherical drop	2.67 ($V= 4/3\pi r^3$)

* (Karrasch *et al.*, 1993)

† This work

The surface of freshly cleaved HOPG imaged in buffer solution (10 mM Tris-HCl, pH 7.5, 150 mM KCl) revealed large smooth terraces (Fig. 2 a). The atomically flat terraces had dimensions up to 2 µm, providing large areas over which adsorbed specimens can be imaged. Scanning a flat area of 200 nm, which is about the scan range for imaging biomolecules at high resolution, a roughness (rms) of 0.02 nm was measured. The carbon atoms of the HOPG could be seen as hexagonally ordered arrays with a lattice constant of 0.23 ± 0.03 nm (Fig. 2 a, inset). The HOPG substrate mounting protocol described above (Materials and Methods, Fig.1 d)

and used for all measurements in this work, was thus good enough to achieve atomic resolution.

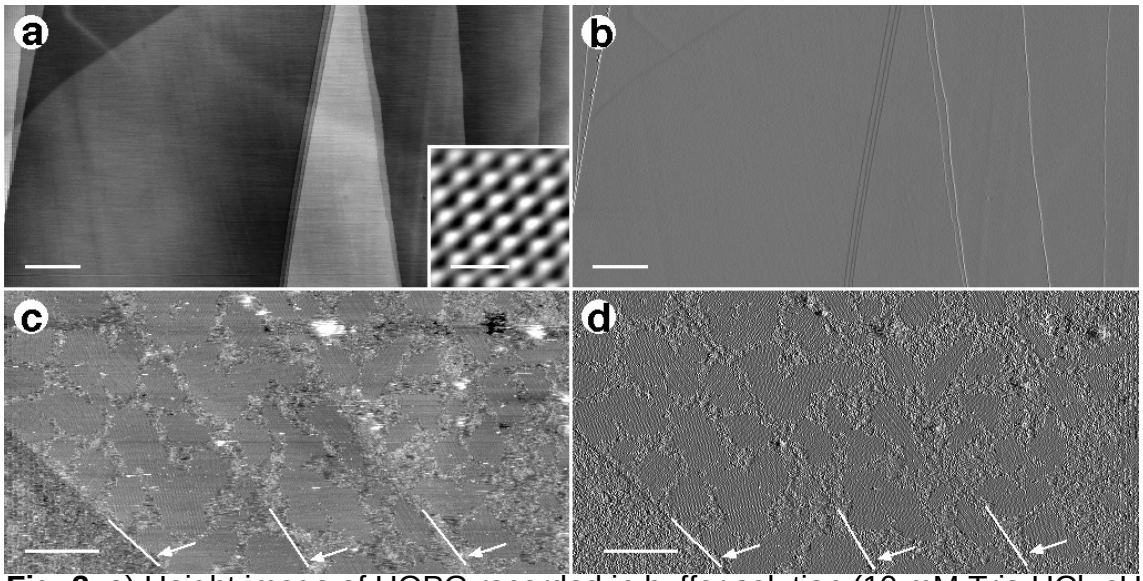

Fig. 2. a) Height image of HOPG recorded in buffer solution (10 mM Tris-HCl, pH 7.5, 150 mM KCl) at minimal force and a scan speed of 4 Hz, showing terraces with atomically flat surfaces (scale bar: 400 nm; full grey scale: 5 nm); Inset: Fourier filtered image of HOPG atoms scanned at minimal force and a scan speed of 12 Hz (scale bar: 5 Å; full grey scale: 2Å). b) Deflection image of a) (scale bar: 400 nm; full grey scale: 1 nm). c) Height image of streptavidin 2D crystals on biotinylated lipid monolayers scanned in buffer solution (10 mM Tris-HCl, pH 7.6, 150 mM KCl) using minimal force and 4.3 Hz scan speed, showing the HOPG terraces (arrows) overlayed by crystalline patches of irregular shape (scale bar: 300 nm; full grey scale: 6 nm); asterisk indicates high plateau. d) Deflection image of c) (scale bar: 300 nm; full grey scale: 1 nm).

Crystallization of streptavidin on biotin-lipid monolayer:

Of many soluble proteins crystallized on lipid monolayers (Brisson *et al.*, 1994), streptavidin is the best-studied simple system to test the suitability of HOPG as a substrate and develop a protocol for the preparation of such crystals for AFM. A lipid monolayer containing biotin lipids was formed on a drop of streptavidin solution and incubated to allow the adsorption and crystallization of the protein (Fig. 1). The appearance of 2D-crystals was inspected by TEM of negatively stained samples and AFM. Many crystals of varying size (80-1500 nm) and shape were found by TEM. Crystals imaged by AFM were in general smaller (50-400 nm), suggesting that crystals adsorbed to the HOPG surface may be disrupted by the unevenness of the substrate. By lowering the speed in approaching the HOPG substrate to the monolayer surface (Fig. 1c), and by cleaving the HOPG with the scotch tape at an angle >120˚, bigger crystals (up to 3 µm) were found apparently adsorbed to the atomically flat surfaces of the terraces (Fig. 2c, d). Selection of high terraces for scanning contributed to the quality of high resolution imaging (see Fig. 2c).

AFM of streptavidin crystals:

The overview AFM micrograph (Fig. 2c, d) shows many crystalline patches of streptavidin 2D-crystals over a scan range of 2.3 µm. The arrows indicate the edges of HOPG terraces that are clearly visible as discontinuities between areas of crystalline streptavidin. Further, breaks between crystal patches on the terraces themselves may indicate that the transfer was influenced by the unevenness of the substrate.

The different crystalline patches typically exhibit different lattice orientations. Between the crystalline patches the protein was not ordered and not well resolved. Imaging streptavidin crystals at medium magnification (Fig. 3a, b) allows single proteins missing within the crystal lattice to be seen (Fig. 3a, arrow 1) and proteins floating away from the crystal edges on the lipid monolayer (Fig. 3a, arrow 2). The crystal in the centre and the left of Fig. 3a had continuous lattice lines, although there were big defects (~100 * ~30 nm) within the crystal. The crystalline patch on the right top of Fig. 3a reveals a different orientation in comparison to the crystal in the centre of the image. Since the appearance of these two crystals was the same tip asymmetries can be excluded. At higher magnification (Fig. 3c) the central region of the big streptavidin crystal is imaged at ~0.8 nm lateral resolution (see diffraction pattern, inset of Fig. 3c). The high signal to noise ratio of the AFM allows every single protein and missing proteins to be resolved (Fig. 3c, arrow 1).

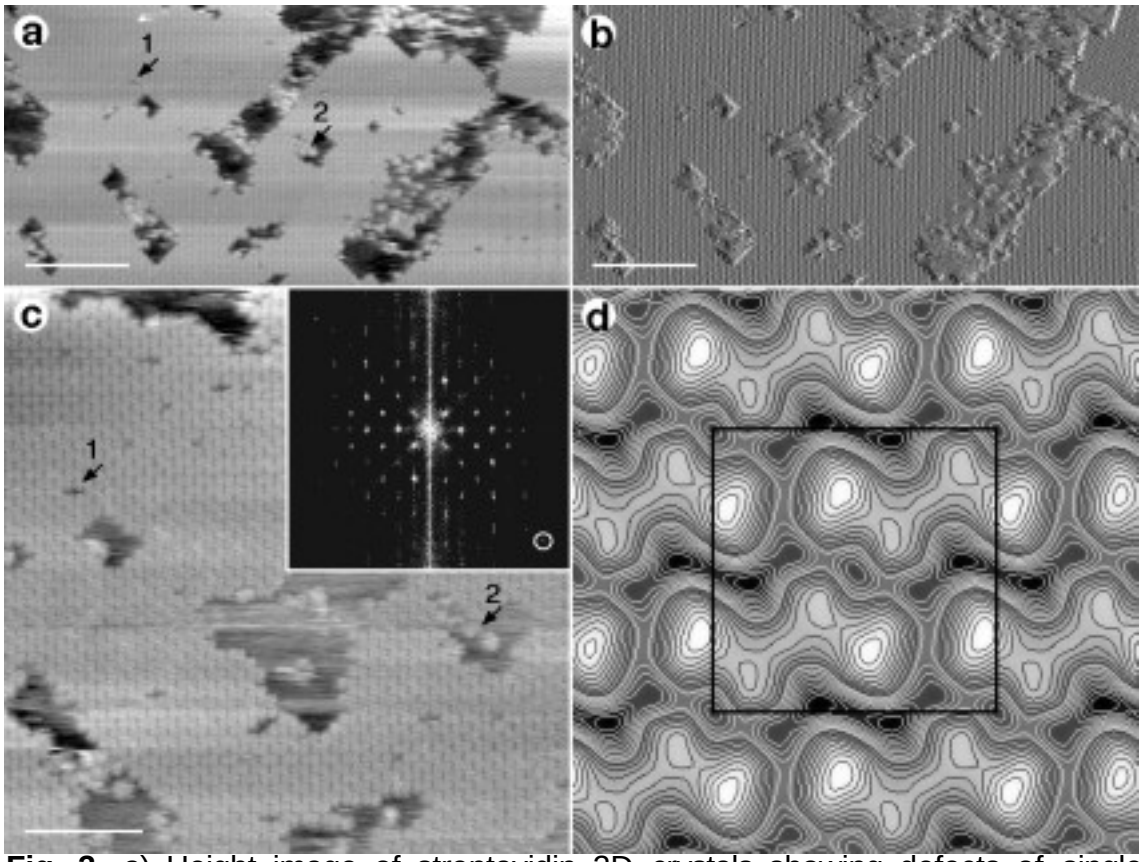

Fig. 3. a) Height image of streptavidin 2D crystals showing defects of single missing proteins (arrow 1) and square shaped edges with losened proteins (arrow 2) scanned in buffer solution (10 Tris-HCl, pH 7.2, 20 mM KCl) (scale bar: 100 nm; full grey scale: 6 nm). b) Deflection image of a) (scale bar: 100 nm; full grey scale: 0.3 nm). c) Height image of the streptavidin 2D crystal of the central region of a) at higher magnification (scale bar: 50 nm; full grey scale: 6 nm), arrows correspond to those in a); Inset: Power spectrum of c) (circle marks spot of 7^{th} order; resolution: 0.83 nm). d) Average over 7 different AFM images using different tips and scan angles (square indicates the unit cell with dimensions of a = b = 8.2 ± 0.2 nm, g = 88 ± 2°; indexed according to Avila-Sakar & Chiu, 1996; full grey scale: 1 nm).

As it is known from other studies (Avila-Sakar & Chiu, 1996; Hendrickson *et al.*, 1989), the streptavidin crystals showed a C_{222} point group symmetry with unit cell dimensions of a = b = 82 Å and g = 90 ° with two tetramers per unit cell (Fig. 3d). In AFM images we found unit cell dimensions of a = b = 84 ± 2 Å and g = 88 ± 2 ° (Fig 3c (inset), d). The height of the crystalline patches above the lipid monolayer were measured as 4.65 ± 0.3 nm (n=20).

TEM of streptavidin crystals:

To examine distortions and other effects the adsorption of the streptavidin crystals to HOPG introduce, the AFM images were compared with low dose electron micrographs of negatively stained streptavidin crystals (Fig. 4). Features up to ~2 nm were resolved (see diffraction pattern, inset Fig. 4a). The unit cell containing two

tetramers were found to have dimensions of a = b = 82 ± 2 Å and g = 90 ± 2 °. In the average from EM (Fig. 4b) each tetramer shows four densities of about equal intensity. These correspond to the two large and two small protrusions in the AFM image (Fig. 3d).

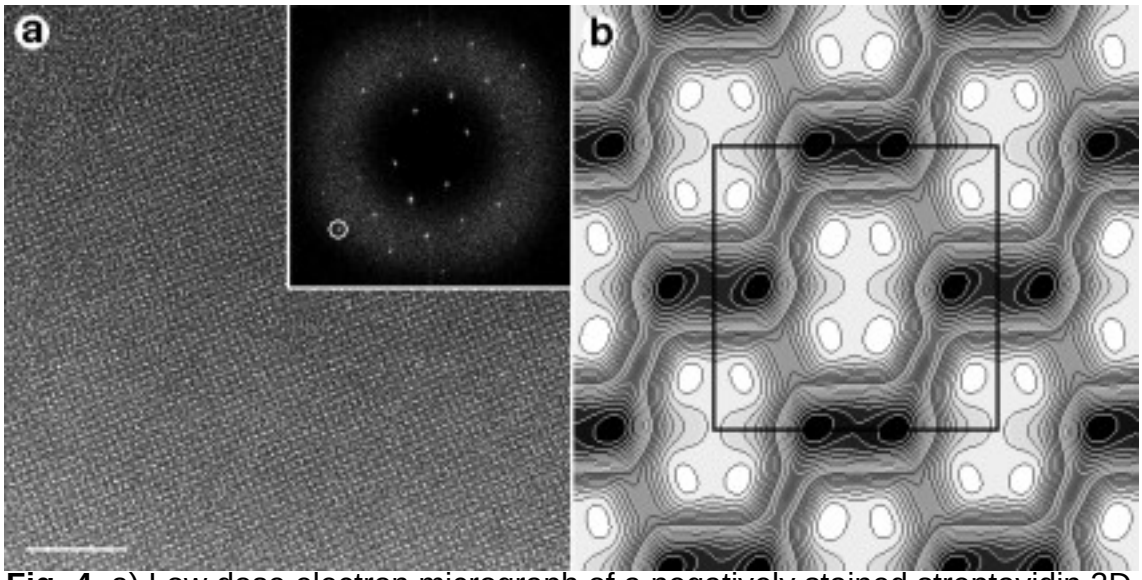

Fig. 4. a) Low dose electron micrograph of a negatively stained streptavidin 2D-crystal (scale bar: 100 nm); Inset: Power spectrum of a) (circle marks spot of 3rd order; resolution: 2.59 nm) b) Average of picture a) (square indicates the unit cell with dimensions a = b = 8.2 ± 0.2 nm, g = 90 ± 2°).

DISCUSSION

In this work we introduce a novel preparation method for high resolution AFM of 2D crystals grown by the lipid monolayer method (Uzgiris & Kornberg, 1983). The streptavidin-biotin lipid system was used as test sample. The fact that we only take advantage of hydrophobic interactions between the fatty acyl chains of the lipid and the pure carbon of the HOPG, indicates the generality of this approach. Interestingly, the difference in hydrophobicity (Table 1) between silanized glass (drop diameter: 4.1 ± 0.2 nm) and HOPG (drop diameter: 3.7 ± 0.2 nm) is crucial for the adsorption of a lipid monolayer prepared as described in this work (Fig. 1). HOPG is a layered material breaking into different layers on cleavage. This can be seen in the AFM images as terraces of varying height and size (up to 2 µm in width) (Fig. 2a, b). Thus, HOPG appears uneven at low magnification, but each terrace is atomically flat, as evidenced by the imaging of individual carbon atoms (Fig. 2a; inset). This also indicates that the substrate is stable enough for high resolution AFM. Feedback loop gains of the instrument can therefore be set high enough for imaging atoms as well as biological samples adsorbed to the substrate. The surface features on the adsorbed streptavidin crystals are not influenced by

abnormalities in the underlying substrate support (Fig. 2, 3). Streptavidin 2D crystals are found to preferentially adsorb to the high plateaus of the HOPG substrate (Fig. 2c, d), which is probably a result of how the contact between HOPG and the monolayer is established during the transfer procedure.

Since streptavidin-biotin is a well known system, different attempts have been made to achieve molecular resolution in the AFM to investigate the biotin binding with the protein. A lipid bilayer was transferred to mica using the Langmuir-Blodgett (LB) and the Langmuir-Schaefer technique. While the first layer consisted only of DPPE (dipalmitoyl phosphatidyl ethanolamine), the second layer was a lipid mixture of biotin-DPPE and DMPE (dimyristoyl phosphatidyl ethanolamine). Streptavidin was added, but the protein did not form highly ordered arrays and molecular resolution could not be achieved (Weisenhorn *et al.*, 1992). Another work reports on the adsorption of streptavidin to a support that was biotinylated after photoactivation of well defined regions. Although the biotinylated sites showed high surface corrugations, only granular features with a diameter of 30 nm could be seen in high magnification images (Mazzola & Fodor, 1995). Furthermore, the biotin-streptavidin interaction was studied by measuring the rupture force of a single biotin-streptavidin bond. To this end, the AFM tip was biotinylated and subsequently streptavidin was adsorbed to it. This tip was approached to a biotinylated agarose bead and retracted. Free biotin binding sites on the streptavidin attached to the tip bound the biotinylated bead and the measured force curve exhibit multiple peaks separated by 160 ± 20 pN, the break force of a single bond (Florin *et al.*, 1994).

With this new preparation method streptavidin can be imaged with the AFM at submolecular resolution. Proteins within the 2D crystals have a high lateral stability as they are supported in the crystallographic packing arrangement. As a consequence they are better resolved than those tetramers floating away from a crystal edge. The slight deviation of the lattice parameters measured on the 2D crystal ($a = b = 84 \pm 2$ Å and $g = 88 \pm 2$ °) from the literature data may either be the result of drift and distortion of the raster scan, or may be caused by the transfer of the sample. The height measured on the streptavidin crystals (4.65 ± 0.3 nm) under appropriate ion conditions (Müller & Engel, 1997) compares favorably to the thickness of the molecule derived from the atomic coordinates (4.3 nm; (Hendrickson *et al.*, 1989)). It is reasonable to assume that the LC-part $(CH_2)_6$ of the biotin-LC-DPPE, which reduces the steric hindrance for streptavidin binding, is the reason of this difference, indicating a good correspondence between the X-ray data and the AFM height analysis.

The unit cell parameters (a = b = 82 ± 2 Å and g = 90 ± 2 °) determined by negative stain electron microscopy are similar to those found by cryo-EM (Avila-Sakar & Chiu, 1996). The small difference between the EM- and AFM-derived unit cell dimensions (see previous paragraph) may be due to the strong adsorption to the HOPG, or to the commonly observed slight drift in the AFM. The former is also manifested in the breakup of the crystal patches (Fig. 2c), compared to electron micrographs where the contiguous crystals are bigger (Fig. 4a). In contrast to the four equal density peaks in the negative stain electron microscopy average representing a projection through the negative stain envelope (Fig. 4b), the AFM data shows a clear difference in height and in shape of the subunits (Fig. 3d). This difference in the appearance of the streptavidin tetramer could be correlated with a surface model derived from the X-ray data. Two subunits (right top and left bottom of the molecule) facing the biotinylated lipid monolayer are resolved as small protrusions, while the two subunits facing towards the tip and exposing the free biotin binding sites (left top and right bottom of the molecule) appear as high protrusions (Fig 3 d). This interesting feature that can be seen in AFM topographs is outlined in Fig. 5.

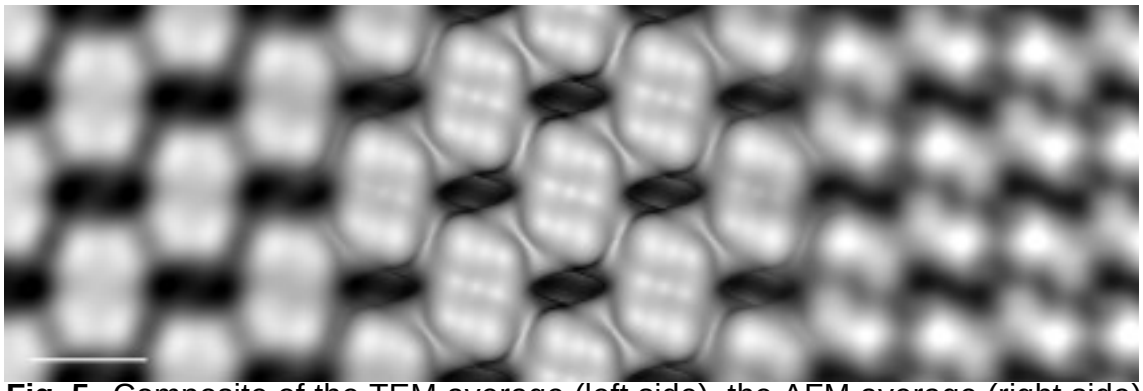

Fig. 5. Composite of the TEM average (left side), the AFM average (right side) and a surface contour model derived from the atomic coordinates (lstp.pdb, Protein Databank) (middle). Because of the C_{222} symmetry of the tetrameric streptavidin molecule, subunits on left top and right bottom are exposed to the AFM tip, while subunits right top and left bottom are more concealed. The EM projection map resolves four equal density peaks, while the AFM surface reveals the differences in access to the subunits from one side of the crystal.

The two free biotin pockets of each tetramer on the top surface of the streptavidin crystal can be used to bind other biotinylated proteins, providing a nanoscale matrix for immobilizing proteins. The flatness of the HOPG surface is essential to minimize undulations in the crystal which would also affect the imaging of bound proteins. The nature of a protein layer bound to the crystal surface is in principle determined by the size and shape of the protein. Proteins of similar size to that of

the streptavidin tetramer or smaller may form a regular packing following the lattice of the streptavidin crystal. Larger proteins would likely arrange in different orientations, but because of the high signal-to-noise ratio of the AFM, may be studied as single particles.

About fifteen proteins have been crystallized into two-dimensional arrays on planar lipid films (Brisson *et al.*, 1994). While some proteins crystallize on a lipid monolayer of charged lipids, a specific interaction is an advantage for binding. The use of Nickel-chelating lipids to bind proteins with a histidine-tag is a promising step to establish a general procedure to crystallize water soluble proteins on lipid monolayers, as demonstrated with a his-tagged reverse transcriptase (Kubalek *et al.*, 1994). Combining a general procedure for crystallization of water soluble proteins on a lipid monolayer and a general preparation method for these specimen for the AFM, a promising avenue is now available for further studies on proteins under native conditions.

ACKNOWLEDGMENT

The work was supported by the Maurice E. Müller foundation of Switzerland, the Swiss National Foundation for Scientific Research (grant 4036-44062 to A. Engel), the Swiss Priority Project for Micro and Nano System Technology, and the French INSERM.

REFERENCES

Avila-Sakar, A. J. & Chiu, W. (1996). Visualization of ß-sheets and side-chain clusters in two-dimensional periodic arrays of streptavidin on phospholipid monolayers by electron crystallography. *Biophys. J.* **70**, 57-68.

Bailey, S. W. (1984). Classification and structures of the micas. Mineralogical Society of America.

Binnig, G., Quate, C. F. & Gerber, C. (1986). Atomic force microscope. *Phys. Rev. Lett.* **56**, 930-933.

Brisson, A., Olofsson, A., Ringler, P., Schmutz, M. & Stoylova, S. (1994). Two-dimensional crystallization of proteins on planar lipid films and structure determination by electron crystallography. *Biol. Cell* **80**, 221-228.

Darst, S. A., Ahlers, M., Meller, P. H., Kubalek, E. W., Blankenburg, R., Ribi, H. O., Ringsdorf, H. & Kornberg, R. D. (1991). Two-dimensional crystals of streptavidin on biotinylated lipid layers and their interactions with biotinylated macromolecules. *Biophys. J.* **59**, 387-396.

Engel, A., Schabert, F., Müller, D. J. & Henn, C. (1995). Imaging membrane proteins in their native environment with the atomic force microscope. Kluwer, Germany.

Florin, E.-L., Moy, V. T. & Gaub, H. E. (1994). Adhesion forces between individual ligand-receptor pairs. *Science* **264**, 415-418.

Green, M. (1975). Avidin. *Adv. Protein chem.* **29**, 85-143.

Hendrickson, W. A., Pähler, A., Smith, J. L., Satow, Y., Merritt, E. A. & Phizackerley, R. P. (1989). Crystal structure of core streptavidin determined from multiwavelength anomalous diffraction of synchrotron radiation. *Proceedings of the National Academy of Sciences, U.S.A.* **86**, 2190-2194.

Jap, B. K., Zulauf, M., Scheybani, T., Hefti, A., Baumeister, W., Aebi, U., Engel, A. (1992). 2D crystallization: from art to science. *Ultramicroscopy* **46**, 45-84.

Karrasch, S., Dolder, M., Schabert, F., Ramsden, J. & Engel, A. (1993). covalent binding of biological samples to solid supports for scanning probe microscopy in buffer solution. *Biophys. J* . **65**, 2437-2446.

Kubalek, E. W., Grice, S. F. J. & Brown, P. O. (1994). Two-dimensional crystallization of histidine-tagged, HIV-1 reverse transcriptase promoted by a novel nickel-chelating lipid. *Journal of Structural Biology* **113**, 117-123.

Mazzola, L. T. & Fodor, S. P. A. (1995). Imaging biomolecule arrays by atomic force microscopy. *Biophys. J.* **68**, 1653-1660.

Moore, A. W. (1973). Chemistry and physics of carbon. Marcel Dekker Inc., New York.

Mou, J., Sheng, S., Ho, R. & Shao, Z. (1996). Chaperonins GroEL and GroES: views from atomic fore microscopy. *Biophys. J.* **71**, 2213-2221.

Müller, D. J., Amrein, M. & Engel, A. (1997). Adsorption of biological molecules to a solid support for scanning probe microscopy. *Journal of Structural Biology* **119**, 172-188.

Müller, D. J. & Engel, A. (1997). The height of biomolecules measured with the atomic force microscope depends on electrostatic interactions. *Biophys. J.* **73**, 1633-1644.

Müller, D. J., Schabert, F. A., Büldt, G. & Engel, A. (1995). Imaging purple membranes in aqueous solution at subnanometer resolution by atomic force microscopy. *Biophys. J.* **68**, 1681-1686.

Ohler, M., Baruchel, J., Moore, A. W., Galez, P. & Freund, A. (1997). Direct observation of mosaic blocks in highly oriented pyrolytic graphite. *Nuclear Instruments and Methods in Physics Research B* **129**, 257-260.

Saxton, W. O. & Baumeister, W. (1982). The correlation averaging of a regularly arranged bacterial cell envelope protein. *J. Microsc.* **127**, 127-138.

Saxton, W. O., Pitt, T. J. & Horner, M. (1979). Digital image processing: Semper system. *Ultramicroscopy* **4**, 343-354.

Uzgiris, E. E. & Kornberg, R. D. (1983). Two-dimensional crystallization technique for imaging macromolecules, with an application to antigen-antibody-complement complexes. *Nature* **301**, 125-129.

Wagner, P., Hegner, M., Kernen, P., Zaugg, F. & Semeza, G. (1996). Covalent immobilization of native biomolecules onto Au(111) via N-hydroxysuccinimide ester functionalized selfassembled monolayers for scanning probe microscopy. *Biophys. J.* **70**, 2052-2066.

Weisenhorn, A. L., Schmitt, F.-J., Knoll, W. & Hansma, P. K. (1992). Streptavidin binding observed with an atomic force microscope. *Ultramicroscopy* **42-44**, 1125-1132.