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Under buffer SFM observation of immunospecies adsorbed on a cyano grafted silicon substrate

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Abstract

Scanning force microscopy (SFM) in contact mode and in liquid medium has been employed to study immunospecies layers adsorbed on a silicon wafer. The silicon wafer has been grafted with a cyanosilane monolayer in order to create a surface with strong adhesive properties which prevent proteins being swept by the scan of the SFM tip. The force curves reveal that the adhesive force has been increased by a factor six without roughness modification (< 1 nm). After the incubation of the surface in a monoclonal antibody (mouse anti-human α-fetoprotein IgG) solution, SFM surface images suggest an homogeneous layer composed by ellipsoidal objects (40–60 nm in diameter, 6–13 nm in height). The substrate was moreover incubated in an antigenic solution (human α-fetoprotein): SFM images reveal that proteins have been added onto the antibody layer.

1. Introduction

Since 1986 the scanning force microscope (SFM) [1] has become an important tool for the study of surfaces of biological interest. Its ability to image surfaces at a molecular level in a water medium appears to be one of the most interesting opportunities in the field of microscopy. Proteins and mainly immunospecies have been considered, either for a better understanding of the protein–protein interaction or for diagnosis application at a molecular level. For such observations, it is necessary to find a substrate able to create a strong enough interaction with the adsorbed proteins to support the high disturbance of the SFM tip, associated with a roughness as low as possible.

During the observation of antibodies physisorbed on mica or polystyrene, the SFM tip sweeps the antibodies, leading to the formation of aggregates after a few scans, because the interactions between proteins and the surface are not strong enough to resist the lateral force induced by the scan of the tip [2–4]. In order to improve the stability, more complex molecular engineered building-up have been employed. Antibodies linked to hapten molecules integrated in Langmuir–Blodgett film [5], a bacterial membrane [6,7] or fixed via a strepdavidin-biotin complex on polystyrene [4] have been studied. Elsewhere antibodies have been imaged using the intermittent contact mode [8] or scan-

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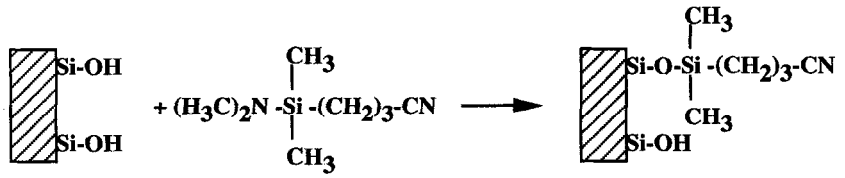

Fig. 1. Reaction of CDDS on a silicon wafer.

ning tunnelling microscopy (STM) [9–11]. Furthermore, roughness modification induced by the formation of antigen–antibody complexes has been pointed out by STM [12] and SFM [13].

The ability of a monolayer to increase the adhesive force without increasing the roughness has been improved with cyano compounds [14]. Elsewhere, silica grafted with cyano compounds is well known to interact with hydroxyl compounds like phenol, carboxylique acid [15] and alcohol [16]. In this paper, we present some results obtained with a new coupling procedure of biospecies onto a silicon wafer grafted with a cyano silane. Using such a method, SFM images in air of IgG-α-fetoprotein complexes have been obtained, leading to ellipsoidal shapes of 50 nm in diameter and 7 nm in height [17]. But in this case, observations in air lead to destructive imaging, due to high contact pressure induced by the capillary force, and was only possible during a short period of time due to deshydratation phenomena.

2. Materials and methods

2.1. Sample preparation

p-Silicon ⟨100⟩ wafers (Microsens, Switzerland) cut in square sample (1×1 cm) was used as substrate. Furthermore, each piece presents a circular central area (3 mm in diameter) covered with a thin thermal silica layer of about 10 nm in thickness (sample 1).

(a) The cyanopropyl dimethyl dimethylamino silane (CDDS) was prepared from dimethylamine and cyanopropyl dimethyl chloro silane (Roth-Sochiel, France) as previously described [18].

(b) As presented by Szabo et al. [19], the grafting process of dimethylamino silane leads to the formation of a monolayer presenting a high coverage density of about 2.6 mol nm^{-2}. The wafers were cleaned with trichloroethylene, acetone, and 2-propanol, hydroxylated with chromic-sulphuric acid, rinsed with pure water and sealed in an ampoule. The ampoule

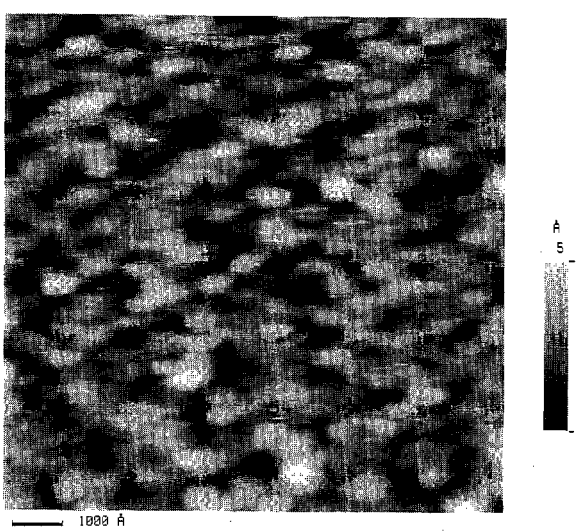

Fig. 2. Typical image of a silicon wafer (sample 1).

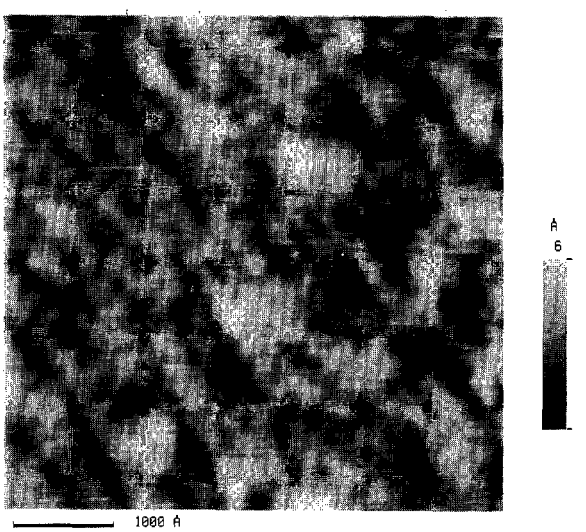

Fig. 3. Image of a silicon wafer after the grafting of a CDDS monolayer (sample 2).

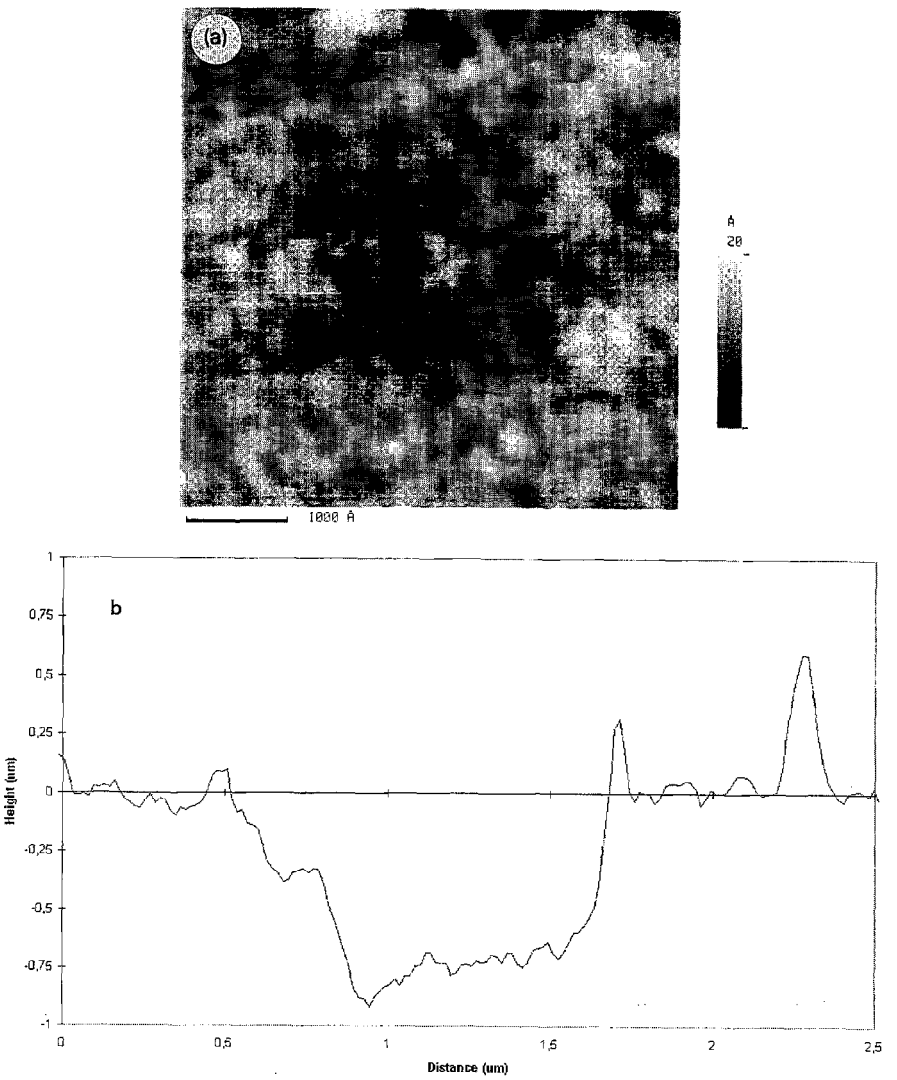

Fig. 4. (a) Image of a grafted silicon wafer (sample 2). The CDDS monolayer in the centre has been removed by a previous scan. (b) Profile obtained on a similar image: The difference in height between the central part and the edge is about 0.6 nm.

was dried by heating at 140°C, under 5×10^{-2} Torr for 120 min. Pure CDDS was then introduced in the ampoule under nitrogen atmosphere. The silanisation was performed in the sealed ampoule under vacuum at 200°C for 24 h. The reaction scheme is given in Fig. 1. The wafer was then washed with tetrahydrofuran and pure water. Finally the dust generated by the grafting process was removed out with a lens cleaning paper (Kodak, USA) moistened with a drop of tetrahydrofuran (sample 2).

(c) 2 μl of a mouse monoclonal anti-human α-fetoprotein IgG (P3F11G9) (BioMérieux, France) solution at a concentration of 2–4 mg ml^{-1} was deposited on the substrate for 24 h at 4°C. The substrate was rinsed three times with a glycine buffer at pH 2.8 (sample 3).

(d) The substrate was moreover incubated one hour in 1 ml of a Phosphate Buffer Saline (PBS)

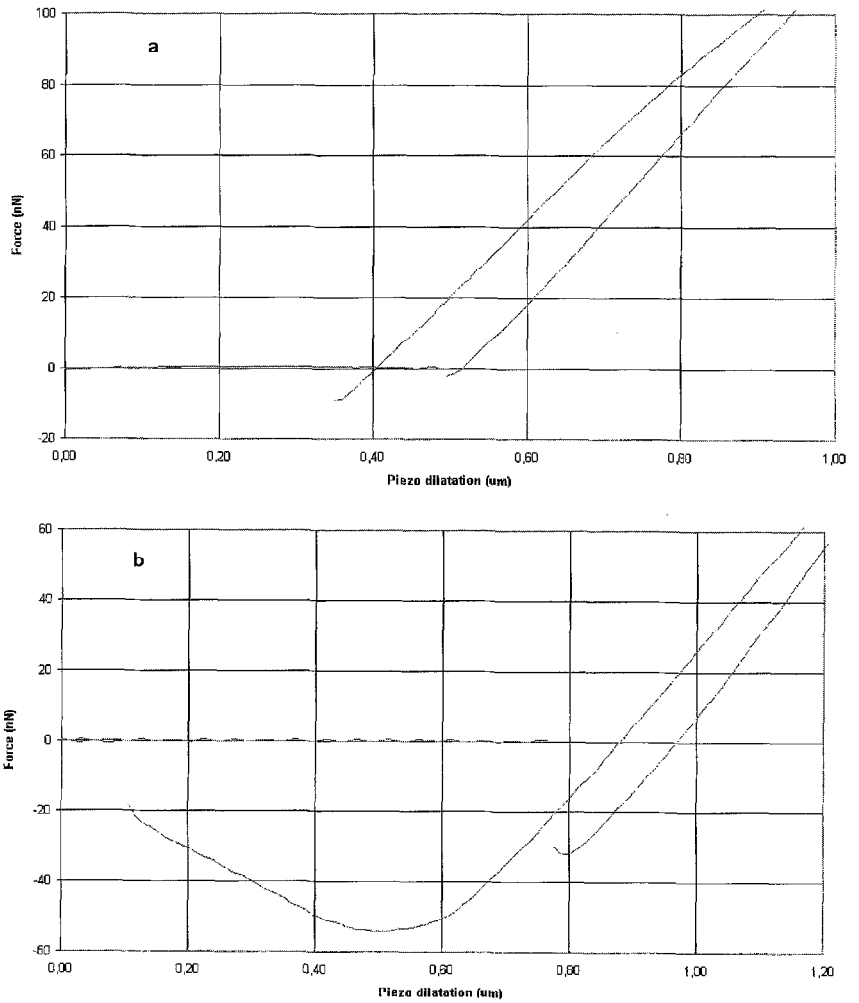

Fig. 5. Typical force curves on (a) silicon wafer (b) CDDS grafted silicon wafer. Force curves give an indication of the nature of the interaction between the tip and the substrate by measuring the cantilever deflection as a function of the piezo dilatation. The adhesion force Fad is obtained by multiplying the force constant of the cantilever (0.21 N m^{-1}) by the distance between the zero applied load and the minimum of the curve.

Tween20 0.05% solution, containing 0.1 ml of horse serum and 2 μl of a 0.34 mg ml^{-1} α-fetoprotein solution (BioMérieux, France). This concentration corresponds to a large excess of antigen (about 1 α-fetoprotein molecule per nm^2). The sample was finally rinsed with a PBS Tween20 0.05% solution (sample 4).

(e) In order to image proteins non-specifically adsorbed on the surface, we incubated a grafted wafer (sample 2) in a horse serum (0.1 ml ml^{-1} in PBS) for 1 h. The surface was washed three times with a glycine solution (pH 2.8) (sample 5).

(f) After imaging the surface, the presence of the antibody and of the α-fetoprotein was confirmed with a classic ELISA test by comparison with a blank surface.

2.2. *Scanning force microscopy*

The SFM data were recorded in constant force mode in a water solution with a commercial SFM [20] using triangularly shaped silicon nitride cantilevers with spring constant of 0.21 N m^{-1} and a tip radius close to 50 nm [20]. Except for Fig. 4 where image was taken in air with a silicon tip (spring constant 0.06 N m^{-1},

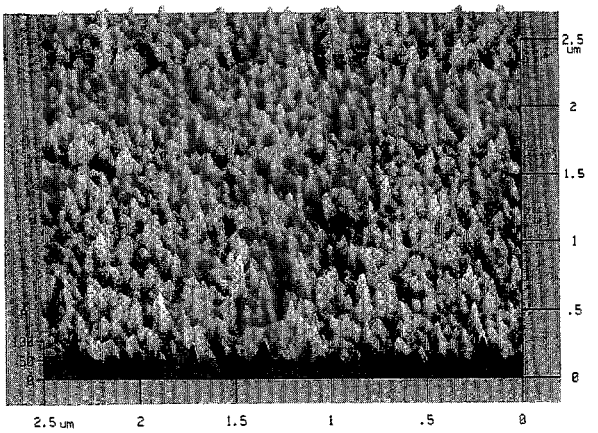

Fig. 6. 3D image of IgG layer (sample 3) taken in PBS: the surface is homogeneously covered by ellipsoidal objets of about 40–60 nm in diameter and 6–13 nm in height.

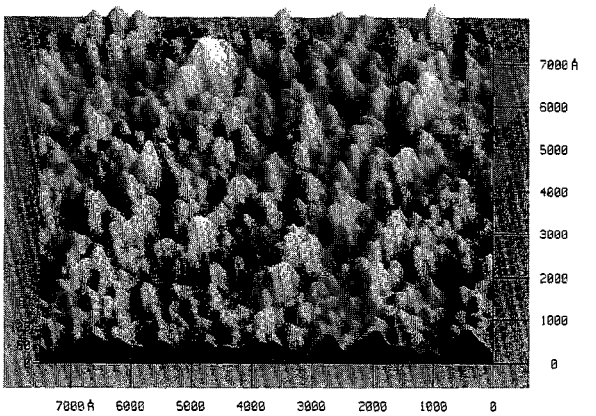

Fig. 7. 3D image of IgG layer (sample 3) taken in an HCl solution pH 2: the IgG are randomly organised on the surface.

tip radius 10 nm) [20]. All the presented images are not filtered.

3. Results and discussion

The silicon wafer is characterised by a low peak-to-valley roughness (0.4–0.8 nm), the surface is typically composed of islands of about 60 nm in diameter (Fig. 2). After the grafting process, significant differences were observed (Fig. 3). The peak-to-valley roughness is slightly affected (0.5–1 nm) and the silane seems to be organised in clusters as previously found [17,21]. Otherwise, we were able to confirm the presence of the silane: The Fig. 4 shows a grafted silicon wafer where the monolayer in the central area has been removed by a previous scan. The difference in height is about 0.6 nm between the central part and the edge, a value which can be compared to the theoretical length of the silane (0.6–0.7 nm).

The force curves have been taken for both surfaces in water (Fig. 5). The maximum adhesion force measured increases from 9 nN for the uncoated wafer to 54 nN for the grafted silicon wafer. The unusual shape of the second force curve is probably due to a capillary force created by a thin layer of hydrolysed CDDS ($HO\text{-}Si(CH_3)_2\text{-}(CH_2)_3\text{-}C{\equiv}N$) confined in the vicinity of the surface. At the minimum of the curve the tip is no more in contact with the surface but is kept close to it by a meniscus of hydrolysed CDDS. The snap-off corresponds to the break of the meniscus. Previously high adhesive forces have already been described with self assembled monolayer of cyano compound in water [14] indicating that strong adhesive properties are probably a characteristic of cyano compounds layer. This force disappears totally after the adsorption of Tween20 or proteins on the surface.

After the adsorption of the antibodies, the surface is covered by an homogeneous layer (thickness 10–12 nm) composed of ellipsoidal objects (Fig. 6). On narrow scale images (Fig. 7), some elements make us think that almost island (6–13 nm in height and 40–60 nm in diameter) is composed of an individual antibody: (i) The diameter of the ellipsoid are close to the theoritical value if we consider the tip-radius induced artefacts [22] (Fig. 11c). (ii) The heights of the islands do not exceed 13 nm, against 14 nm for the maximum length of an IgG. (iii) Almost no island has more than one maximum in height. (iv) The density of the ellipsoid (660 per μm^2) is low compared to the maximum possible coverage of an antibodies layer (20000 IgG μm^{-2}). Furthermore the difference in height could be interpreted as a random configurations of the antibodies (Fig. 8).

After incubation of the antibodies with the antigenic solution, large scale images of the surface show a very heterogeneous surface. "Macro proteins" are clearly visible on the surface (Fig. 9). The thickness of the protein layer reaches to 200 nm. Narrow scale images show that the surface is locally composed of proteins adsorbed over the antibodies (Fig. 10). The height of such complexes is about 15–22 nm, a value inferior

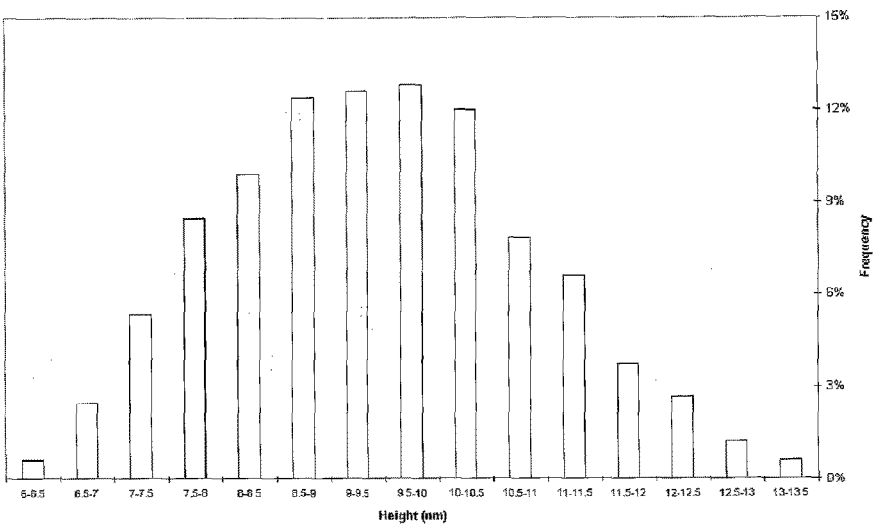

Fig. 8. Distribution of the height of the ellipsoid. Note the Gaussian profile of the curve indicating different configurations of the IgG.

to the maximum height of an α-fetoprotein-IgG complex supposed to be 22 nm (Fig. 11). We are not able to know if this increase in height is due to an antigen–antibody interaction or to a non-specific adsorption. Nevertheless, we know that proteins passively adsorbed on the surface are removed easily by the scan of the SFM tip, and we could imagine that after a few scans only the antigen–antibody complexes are still fixed on the surface.

However, imaging serum proteins adsorbed on a grafted wafer leads to the formation of a layer with a thickness of about 10–15 nm indicating that the serum proteins do not tend to aggregate on the surface (Fig. 12).

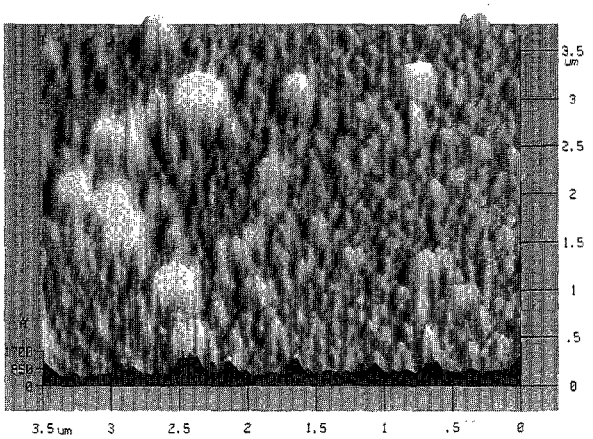

Fig. 9. Wide scale 3D image of IgG incubated in an antigenic serum taken in PBS Tween20 0.05% (sample 4). The surface is heterogeneous, an important number of wide objects are adsorbed on the surface.

4. Conclusions

We have demonstrated that cyano silane monolayers increase the adhesive properties of a silicon wafer, and

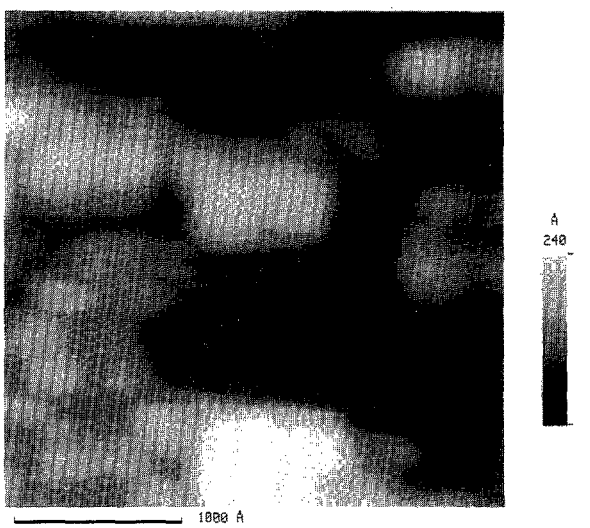

Fig. 10. Narrow scale image of IgG incubated in an antigenic serum taken in a PBS Tween20 0.05% (sample 4). The image shows clearly that a second layer (white) had been adsorbed over the antibodies layer (light grey).

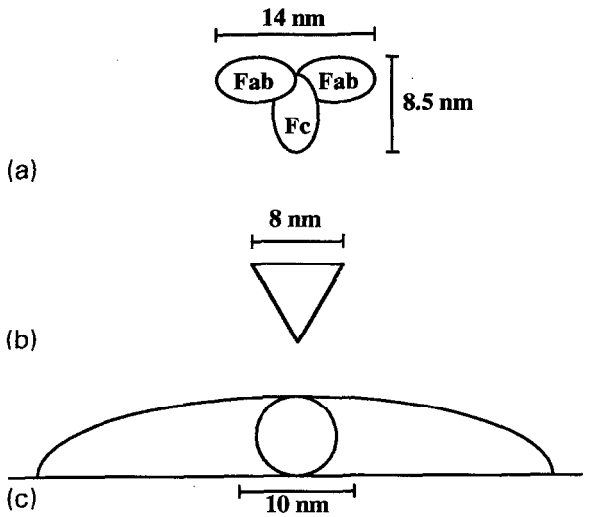

Fig. 11. Dimensions of (a) the IgG monoclonal antibody [23] and (b) of the α-fetoprotein [24] as determined by X-ray diffraction. The thickness of both proteins is 3 nm. (c) SFM topographic profile of one IgG using a tip with a radius of 50 nm [22]. The IgG (mean geometric diameter = (length × height × thickness)$^{1/3}$ = 7 nm) would have a profile of 53 nm (shape radius = 2 × (tip radius × IgG radius)$^{1/2}$ = 26.5 nm) in width.

the ability of imaging protein layer adsorbed on such a surface by SFM in a water medium.

The ability of imaging immunospecies in a buffer is of great interest for the understanding of the phenomena implicated in the immunoassays. The organisation and the function of the antibodies, the antigen–antibody reaction, the adsorption–desorption of proteins could be studied in real time.

The differences observed before and after the incubation of the antigen show that it may be possible to detect individual antigen–antibody complexes. As previously described [13], this suggests that SFM could be used as an immunoassay for detecting very low concentration of antigen.

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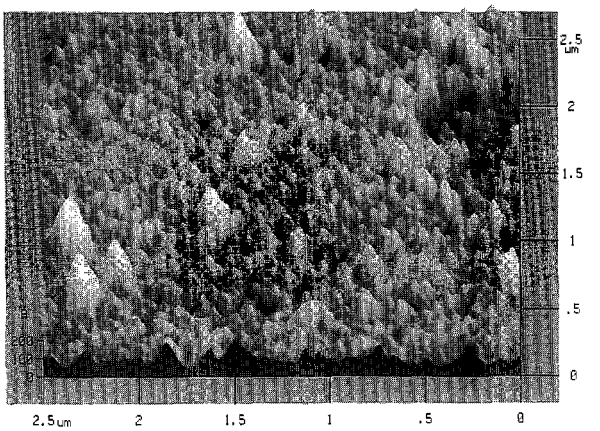

Fig. 12. 3D image of serum proteins taken in PBS (sample 5). The average thickness of the protein layer is 10 nm.

References

[1] G. Binnig, C.F. Quate and C. Berger, Phys. Rev. Lett. 56 (1986) 930.
[2] J.N. Lin, B. Drake, A.S. Lea, P.K. Hansma and D.J. Andrade, Langmuir 6 (1990) 509.
[3] A.S. Lea, A. Pungor, V. Hlady, D.J. Andrade, J.N. Herron and E.W. Voss, Langmuir 8 (1992) 68.
[4] J. Davies, C.J. Roberts, A.C. Dawkes, J. Sefton, J.C. Edwards, T.O. Glasbey, A.G. Haymes, M.C. Davies, D.E. Jackson, M. Lomas, K.M. Shakesheff, S.J.B. Tendleer, M.J. Wilkins and P.M. Williams, Langmuir 10 (1994) 2654.
[5] A.L. Weisenhorn, H.E. Gaub, H.G. Hansma, R.L. Sinsheimer, G.L. Kelderman and P.K. Hansma, Scanning Microsc. 4 (1990) 511.
[6] P.J. Mulhern, B.J. Blackford, M.H. Jericho, G. Southam and T.J. Beveridge, Ultramicroscopy 42–44 (1992) 1214.
[7] F. Ohnesorge, W.M. Heckl, W. Häberle, D. Pum, M. Sara, H. Schindler, K. Schilcher, A. Kiener, D.P.E. Smith, U.B. Sleytr and G. Binnig, Ultramicroscopy 42–44 (1992) 1236.
[8] P. Warkentrin, B. Wälivaara, I. Lundström and P. Tengvall, Biomaterials 15 (1994) 786.
[9] R.J. Leatherbarrow, M. Stedman and T.N.C. Wells, J. Mol. Biol. 221 (1991) 361.
[10] C.H. Olk, J. Heremans, P.S. Lee, D. Dziedzic and N.E. Sargent, J. Vac. Sci. Technol. B 9 (1991) 1268.
[11] J. Rocca-Serra, J. Thimonier, J.P. Chauvin and J. Bardet, J. Vac. Sci. Technol. B 12 (1994) 1490.
[12] J. Masai, T. Sorin and S. Kondo, J. Vac. Sci. Technol. A 8 (1990) 151.
[13] L. McDonnell, E.M. Cashell, J. O'Mullane, S. Fanning, M. Delaney, J. Snauwaert and L. Hellemans, in: Surface Properties of Biomaterials, Eds. R. West and G.Batts (Butterworth-Heinemann, UK, 1994) p. 145.
[14] H. Yamada, S. Akamine and C.F. Quate, Ultramicroscopy 42–44 (1992) 1044.

[15] M.J. Bertrand, S. Stefanidis and S. Sarrasin, J. Chromatogr. 351 (1992) 164.
[16] F. Gardies, Thèse de Doctorat, Ecole Centrale de Lyon, 1990.
[17] C. Saby, Thèse de Doctorat, Ecole Centrale de Lyon, 1993.
[18] Y. Duvault, A. Gagnaire, F. Gardies, N. Jaffrezic-Renault, C. Martelet, D. Morel, J. Serpinet and J.L. Duvault, Thin Solid Films 185 (1992) 2219.
[19] K. Szabo, N. Le Ha, P. Scheinder, P. Zeltner and E. Kovats, Helv. Chim. Acta 67 (1984) 2128.
[20] Park Scientific Instruments, Sunnyvale, CA, USA.
[21] L. Bourdieu, M. Maaloum, P. Silberzan, D. Ausserre, G. Coulon and D. Chatenay, Ann. Chim. Fr. 17 (1992) 229.
[22] M.J. Allen, N.V. Hud, M. Balooch, R.J. Tench, W.J. Siekhaus and R. Balhorn, Ultramicroscopy 42–44 (1992) 1095.
[23] E.W. Silverton, M.A. Navia and D.R. Davies, Proc. Natl. Acad. Sci. 74 (1977) 5140.
[24] X.M. He and D.C. Carter, Nature 358 (1992) 209.