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Adsorption of proteins to fused-silica capillaries as probed by atomic force microscopy

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Abstract

In order to prove binding of proteins to the capillary wall, the inner surface of naked silica has been probed with the aid of atomic force microscopy. A large protein (ferritin, a particle of 12 nm diameter) has been left in contact with the capillary dissolved in buffers both below (pH 4.6) and above (pH 7.0) its p*I* (5.0–5.2) value. The capillary was then sliced lengthwise and its surface explored with the atomic force microscopy tip. Massive protein adsorption onto the naked fused-silica wall was observed, both below and above the protein p*I*, the thickness and extent of such deposition being proportional to the initial concentration of the protein bathing the wall. Such proteinaceous material could be largely desorbed by washing the capillary in 1 *M* NaOH, this process restoring the original topography of naked fused-silica. Additionally, such binding was also demonstrated electrophoretically by a displacement process which consisted of desorbing the bound ferritin by driving anionic detergent micelles (sodium dodecyl sulphate) from the cathodic compartment. Atomic force microscopy could thus become a powerful tool for probing surface adsorption also to coated capillaries, thus helping in designing better, more hydrophilic coatings.

Keywords: Atomic force microscopy; Adsorption; Capillary columns; Silica surface; Proteins

1. Introduction

It is well known that sample adsorption onto the fused-silica wall of a capillary by means of ionic, hydrophobic and/or other mechanisms of interaction significantly reduces the efficiency of an electrophoretic separation (e.g., [1–5]). In particular, the effect of adsorption is to change the sample concentration profile, the velocity of the zone motion and the amount of substance in the analyte zone. In addition, in the case of polyelectrolytes, and especially of polycations, adsorption to the wall can induce charge reversal and thus change the direction of the electroosmotic flow [5]. A number of articles, devoted to the theoretical treatment of these phenomena, have appeared (see, as a selection, [5–12]). Most of them adopted mathematical models which are related, to variable extents, to those of chromatography (for reviews, see [13,14]). Such sample–wall interaction can either be linear (e.g., [7–11]),

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when the rate of sample adsorption is proportional to its concentration, or non-linear (e.g., [5,12–14]), when the Langmuir adsorption isotherm is used and the sorption of the substance is proportional to its concentration.

An impressive array of methods for quenching or completely abolishing analyte interaction with the silica wall has been offered: buffer changes and additives; use of organic solvents; adsorption of neutral and/or charged macromolecules (including surfactants) to the wall; chemically bonded phases (for extensive reviews, see [15,16]). It is generally agreed today that the best way to shield silanol groups and thus prevent protein adsorption is via polymeric layers, either adsorbed or (preferably) covalently bound to the wall, as originally proposed by Hjertén [2]. Among such polymers, polyacrylamide is quite popular, due to the ease of the reaction for attachment to the wall. However, hydrolysis-resistant, *N*-substituted polyacrylamides, such as *N*-acryloylaminoethoxyethanol, are to be preferred [17], since the facile hydrolysis of the amido bond of pure acrylamide, even at moderately alkaline pH values, regenerates a negative charge on the wall. Such coatings have a two-fold effect: (a) they impede access of macromolecules to the silica wall, due to their very high viscosity and thoroughness of cover (only if properly executed!); (b) they effectively obliterate the ionizable silanol groups, since such groups are used for covalent attachment of the polymer via a bifunctional agent (however a lot of exceptions apply, depending on the type of anchoring agent; see, e.g., [18]).

However, many reports also exist in the literature which claim that the electroosmotic flow may be controlled and, as a result, that protein binding to the wall may be reduced or abolished simply by manipulating the background electrolyte bathing the "naked" silica surface (given an average pK value of 6.3, a few silanols will already be ionized starting with pH 2.3 [19]). Typically, diamines are utilized for this purpose: e.g., putrescine [20,21], spermine [22], 1,3-diaminopropane [23], 1,5-diaminopentane [24], ethylenediamine [25], a whole series of amines (such as propylamine, pentylamine, dipropylamine, dipentylamine) [26] and also amino sugars [27]. In addition, it is believed that, simply by running proteins at a buffer pH well above the isoelectric point (pI) of all components in the mixture (where they will exhibit a net negative charge) adsorption will be abolished [28,29].

In the present report, we use the atomic force microscopy (AFM) to probe the adsorption of proteins onto the capillary wall. AFM is essentially a scanning method that provides topographical maps of a surface [30]. A very sharp tip, about 5 μm long, with a typical end radius r of ca. 10 nm scans the surface. The tip is located at the free end of a cantilever which bends or deflects as the tip moves on the surface. An optical detector measures this deflection, which has a relationship with the surface morphology. The experimental data thus obtained are processed by a computer and stored in a two-dimensional array in which each element corresponds to a point of the surface and whose value gives the height of the surface in that point. In such a way, the AFM measures topography by mechanically moving this sharp probe across the sample to "feel" the asperities of the surface, similar to the way a phonograph stylus was used to navigate the grooves of old vinyl records. AFM has been recently shown to be a valuable tool in life science research [31].

The paper is organized as follows: in the first section of the results, we probe protein binding at low and high protein loads at pH values below the pI; subsequently, the capillary is probed again after desorption with NaOH. In the second section, protein adsorption is probed, at low and high sample loads, at pH values above the pI, followed by re-probing after desorption. Finally, the existence of protein bound to the wall is verified by direct measurements in zone electrophoresis, by frontally sweeping bound material with anionic detergent that has been electrophoretically transported into the capillary from the cathodic compartment.

2. Materials and methods

2.1. Reagents

Recombinant DNA ferritin (a large, iron storage protein of M_r 440 000, a spherical particle of ca. 12 nm diameter) was a kind gift from Dr. P. Arosio, S. Raffaele Hospital, Milano, Italy [32]. Fused-silica

capillaries (100 μm I.D.×375 μm O.D.) were from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Protein adsorption on the capillary surface

The capillaries were first washed with detergent, then with 1 *M* NaOH, followed by rinses in distilled water, then with 1 *M* HCl, with final rinses until a neutral pH was obtained. After drying in a currrent of nitrogen, adsorption experiments were performed as follows. In one set, the capillaries were equilibrated with 30 m*M* Tris–acetate buffer, pH 4.6, i.e. a pH value just below the p*I* (5.0 to 5.2) of ferritin. Then low (1.6 mg/ml) and high (16 mg/ml) concentration solutions of ferritin, in pure water, were injected with a syringe. The capillaries were left standing at 4°C overnight, then thoroughly washed with distilled water and dried under a gentle stream of argon. In another set of experiments, the same protein loading was performed, but with the capillaries and protein equilibrated in 30 m*M* sodium–phosphate buffer, pH 7.0, i.e. at pH values well above the p*I* of ferritin.

2.3. Electrophoretic protein desorption

In order to confirm protein binding with an independent technique, in some experiments the capillaries were filled with high protein loads (16 mg/ml) under a microscope, just up to, and before, the detection window. After excess protein solution had been flushed away, desorption was effected in the electric field by adding 30 m*M* sodium dodecyl sulphate (SDS) to the previously-mentioned pH 4.6 and pH 7.0 buffers, but only to the cathodic compartment and transporting it electrophoretically towards the detector (placed at the anodic side). This could be described as the electrophoresis analogue of displacement chromatography, where a substance that has a stronger affinity than any of the feed components for the stationary phase is pumped into the column [33]. CZE was performed with a Bio-Rad (Hercules, CA, USA) BioFocus 2000 instrument.

2.4. Cutting of the capillary for AFM analysis

Short segments of capillaries, pretreated with ferritin solutions at pH 4.6 and at pH 7.0, were cut out. The polymide coating was removed by scraping and the capillaries were glued to a glass square with epoxy resin. Using a diamond point cutter, two incisions were made lengthwise in opposite sides of the cylindrical surface. The upper half of the cylinder was then removed by simple expulsion with a very sharp tip cutter. Finally, one of the extremities was bevelled, so as to gain access to the floor of the half cylinder for the AFM tip (see Fig. 1). All operations were performed under an optical microscope Stemi 2000 from Zeiss (Oberkochen, Germany). An Axioskop microscope from Zeiss (with a 200 magnification factor) was then used for the picture in Fig. 1. This image was acquired with a videosystem, digitized in a personal computer and elaborated under the program Corel Draw.

2.5. Atomic force microscopy

The scanning probe microscope was from Park Scientific Instruments (1171 Borregas Ave., Sunnyvale, CA 94089, USA). A small capillary chip was mounted on the platform of the AFM instrument and scanned both in the contact mode (i.e., with the AFM tip making a soft physical contact with the sample, also known as repulsive mode) and in the noncontact mode (also known as attractive mode. in

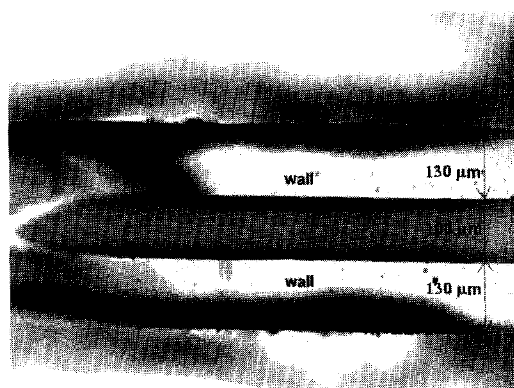

Fig. 1. Picture of the capillary cut lengthwise. Note the bevelled left extremity, allowing the AFM tip (of only 5 μm length) to probe the bottom of the cylindrical cavity. Picture taken with a Zeiss microscope under polarized light at a 200-fold magnification. The greyish area outside the capillary image is a layer of glue.

which the AFM monitors attractive Van der Waals forces between the tip and the sample). The two methods gave similar results, but the data presented are those taken in the contact mode only, since in this mode the surface is probed with higher resolution. Data were obtained on a 256×256 points digitizing grid. We have chosen two sizes of the scanned area: 1.5×1.5 and 2×2 μm.

3. Results

The experiments evolved along two lines: in the first, the potential protein adsorption was monitored with AFM; in the second, the same phenomenon was explored by direct desorption with SDS in a capillary under an electric field. It was first necessary to probe the quality of the capillary cutting in order to be sure that the tip of the AFM instrument would be exploring the inner silica surface. This is shown in Fig. 1: the cut walls of the capillary and the inner, half cylindrical surface, are clearly visible. One should also note the left extremity, cut to a slant, to form a "flute beak": without this precaution, the AFM tip (which is only 5 μm long) could never make a good contact with the bottom of the half cylinder which is surrounded by walls as high as 80 μm. Thus, probing with the AFM tip occurred only on the extreme left side of this image.

3.1. Probing protein adsorption at pH 4.6

In a series of experiments, ferritin adsorption was assessed at pH 4.6, i.e. a buffer pH value just below the p*I* of the protein, where the silica wall would be about 5% negatively charged and the protein possess a slight excess of positive charge. Fig. 2A–C shows the aspect of the capillary surface, prior (2A) and after exposure to 1.6 mg/ml (2B) and 16 mg/ml (2C) ferritin. It can be appreciated that the surface topography of the naked capillary is drastically changed in the presence of ferritin, as though it had been blanketed with a veil of material, thinner and with larger "holes" in the case of more dilute ferritin solutions. This can be better visualized in the three-dimensional pictures of Fig. 3. In Fig. 3a, the surface of a naked capillary is explored: it shows the typical average rugosity of ca. 5 nm, as previously reported by us [34]. In Fig. 3b (1.6 mg/ml ferritin) the original surface topography appears to be completely obliterated by a veil of material, thin in most regions, but forming "peaks" as high as 60 nm in several regions of the surface. In Fig. 3c (16 mg/ml ferritin) this veil appears to be more uniform in thickness and in distribution over the surface. Given the fact that it could extend 25 to 40 nm above the original "rugosity" of the naked surface, and that the shape of the surface structure in the deepest holes recalls the starting surface roughness of the naked capillary, it is tempting to speculate that protein might have been deposited in two or three layers.

3.2. Probing protein desorption at pH 4.6

In order to prove whether the "vellum" we see on the surface could be proteinaceous material, we have attempted to rub it off with NaOH. The probed half cylinder was gently filled with a few microlitres of 1 *M* NaOH, with the aid of a micro syringe. After incubation for 30 min, the capillary was washed with distilled water and this operation was repeated three times. Blotting was done by capillary forces with the aid of a cotton swab, so as not to scrape the surface accidentally. After drying in a stream of nitrogen, the same surface was probed again with AFM. As shown in Fig. 4, the surface now appears similar to the control images of Fig. 3a, with the typical average rugosity of 4–5 nm (in this particular picture, the surface is cut by a shallow valley in the middle, with a depth of ca. 2 nm). We have also probed several other regions, in order to see whether this picture was valid over a much wider surface. This was found to be the case; however, occasionally, we still found ridges of proteinaceous material which, due to the slow diffusion process in the static NaOH solution, had not been completely rubbed off (not shown).

3.3. Probing protein adsorption at pH 7.0

This is indeed a more interesting case because, when the pH is well above the p*I* value, proteins should possess a high enough net negative charge to ensure repulsion from the wall. As suggested by our data, however, this does not seem to be the case. Fig. 5a shows again the naked inner capillary surface, equilibrated at pH 7.0 in the absence of protein. The

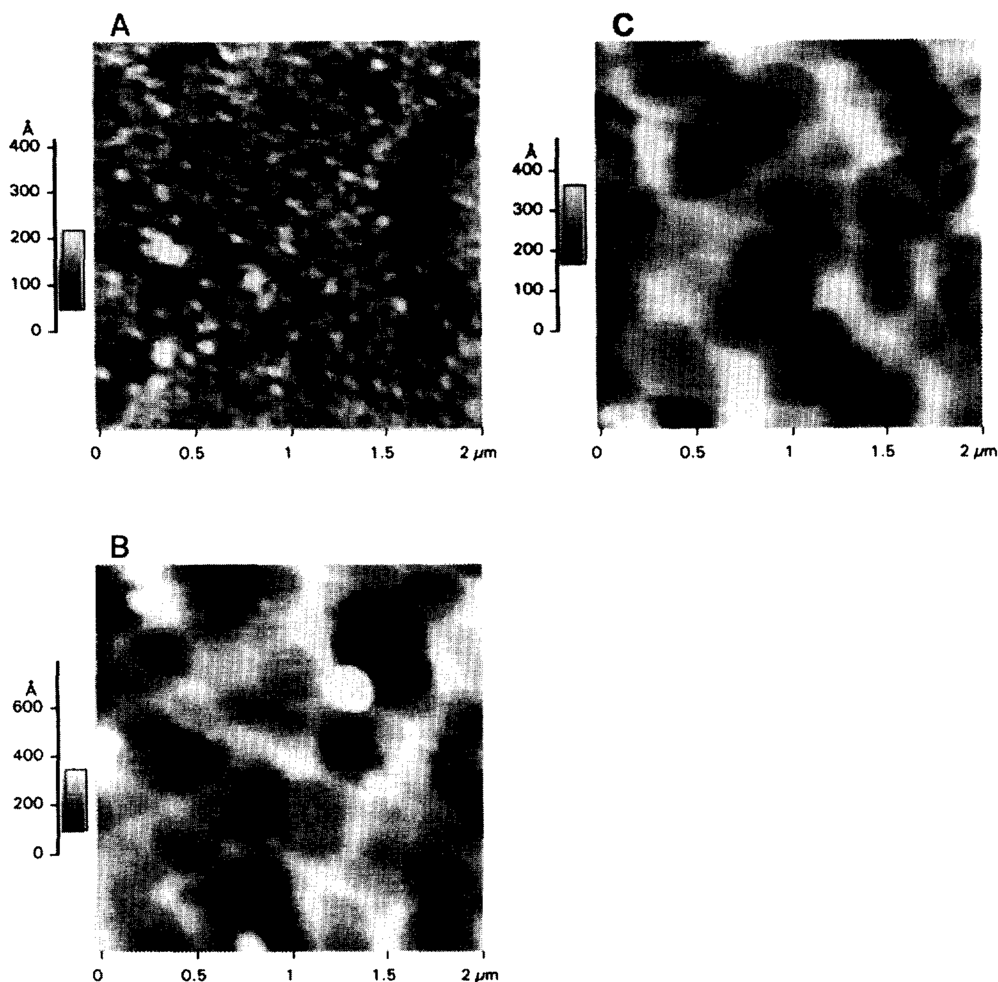

Fig. 2. Scan of a 2×2 μm surface area of the inner wall of a fused-silica capillary. (A) Control; (B) after adsorption of a 1.6 mg/ml ferritin solution at pH 4.6; (C) after adsorption of a 16 mg/ml ferritin solution at pH 4.6. Note, in the last two cases, the complete obliteration of the original surface morphology. Note additionally that the white areas represent peaks and dark areas valleys (probably reaching the floor, i.e. the bare silica surface).

topographic features are very much the same as in Fig. 3a. If a ferritin solution of 1.6 mg/ml is then left in contact with the wall, the picture of Fig. 5b is obtained: while the surface appears to be largely devoid of proteins, occasional peaks are found on the 2×2 μm surface explored: a 60 nm tall peak can be in fact seen midway on the extreme left side; occasional ridges are also found scattered on the plane. The picture changes drastically if the concentration of ferritin in the capillary is raised to 16 mg/ml. Now, as seen in Fig. 5c, a protein "vellum" appears to blanket the entire surface, much like in the experiments performed at pH 4.6, with perhaps thinner areas and larger holes devoid of protein. Thus, it would appear that adsorption is proportional to the initial protein load, as would be expected if we assume Langmuir's isotherms.

3.4. Probing protein desorption at pH 7.0

In a similar manner to the experiments at pH 4.6, the cut capillaries were incubated with 1 *M* NaOH and the potential desorption of deposited material probed again. As shown in Fig. 6a and b, ferritin

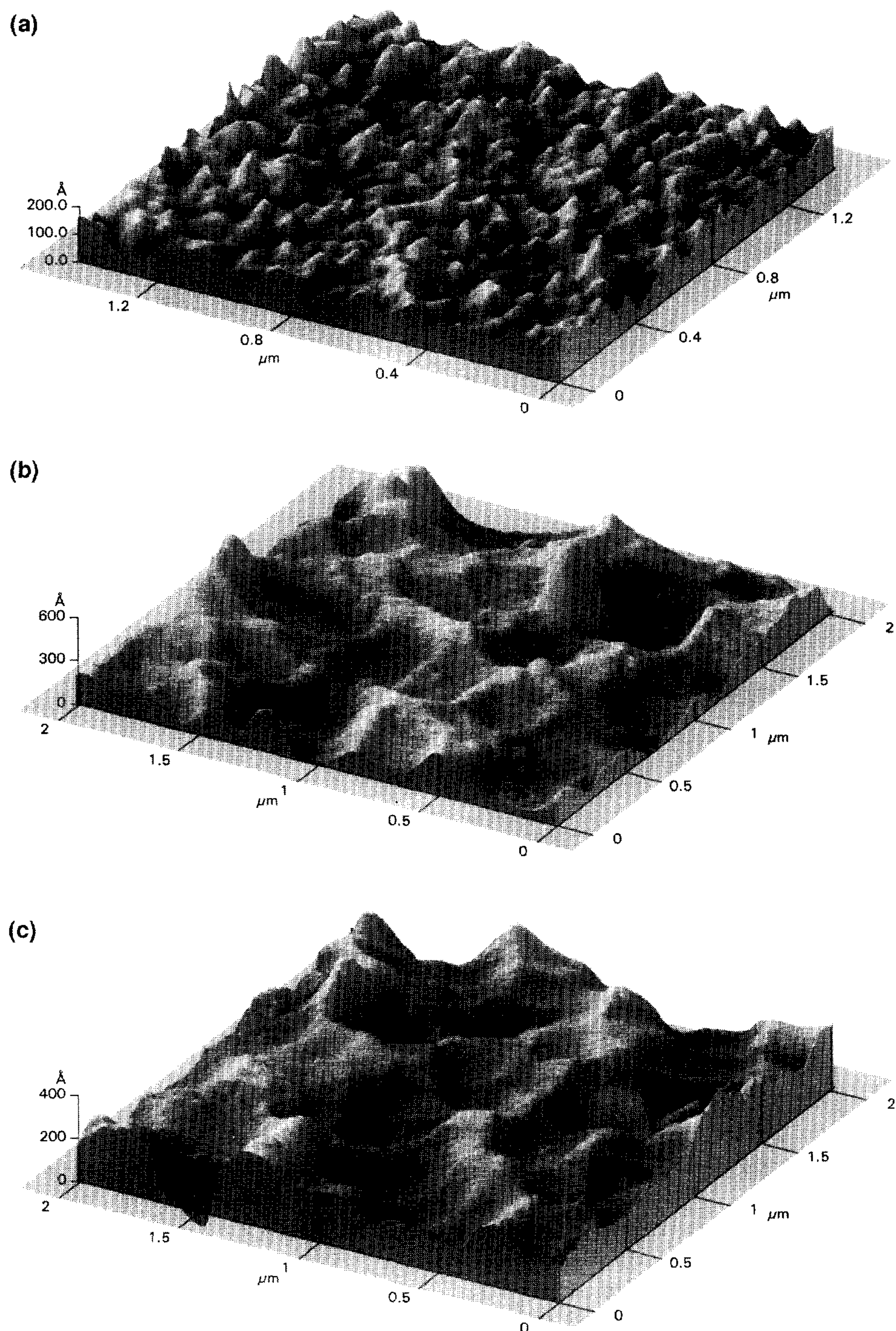

Fig. 3. Three-dimensional representation of the surface area of the 2×2 μm scans in Fig. 1. (a) Control; (b) after adsorption of a 1.6 mg/ml ferritin solution at pH 4.6; (c) after adsorption of a 16 mg/ml ferritin solution at pH 4.6. Note that the original surface rugosity of a naked capillary is of the order of 4–5 nm, with peaks not greater than 10 nm. In (b) and (c), the original surface topology is completely blanketed out by a veil of proteinaceous material reaching a total thickness as great as 50–60 nm.

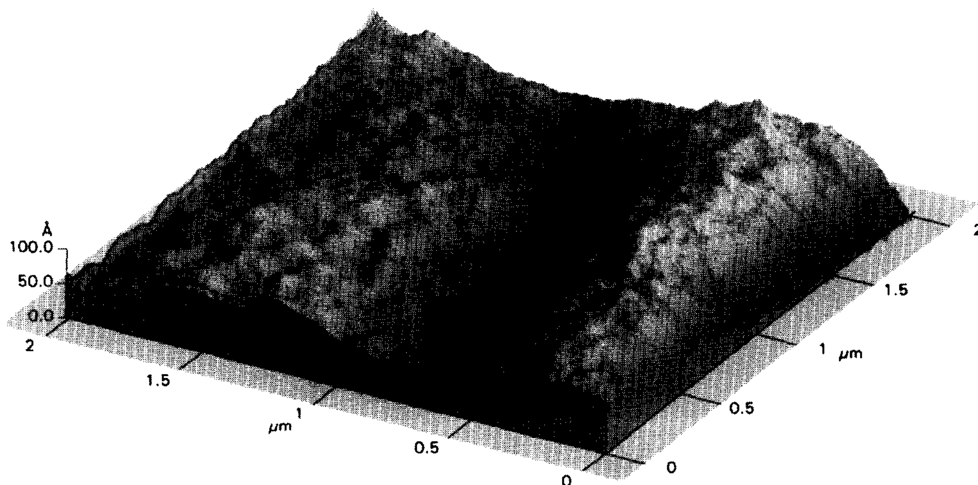

Fig. 4. Protein desorption from the silica surface. After incubation in 1 *M* NaOH of the same silica chips shown in Fig. 3b and Fig. 3c, the surface was probed again by AFM. Note how the original surface rugosity of plain silica, in the absence of protein, is restored.

seems to be efficiently removed from the surface, which now appears smooth, at low protein loads; at high loads (16 mg/ml) a few peaks appear still scattered on the plain, but the surface has been largely cleared of deposited material (cf. Fig. 5c).

3.5. Probing protein binding by capillary electrophoresis

An independent assessment of protein binding would be to prove it by direct electrophoretic experiments. This could be done, in capillaries in which ferritin had been adsorbed at pH 4.6 and pH 7.0, by a kind of "displacement" electrophoresis, by using anionic detergents as displacers. It is well known that SDS has a strong affinity for proteins and forms negatively charged micelles into which the protein moiety is sequestered [35]. SDS thus acts by equalizing the charge/mass ratio in proteins, which then behave, electrophoretically, in a similar manner to DNA coils. Thus, a sweeping wave of SDS should be able, in principle, to detach proteinaceous material that was previously bound to the capillary wall. As shown in Fig. 7, this seems to be the case: a "wave" of released protein is seen migrating with the SDS front, emerging at the anodic side of the capillary and transported electrophoretically from the cathodic compartment. This occurs both below (pH 4.6, Fig. 7a) and above (pH 7.0, Fig. 7b) the p*I* value of ferritin. Interestingly, a second run releases a small amount of protein (ca. 5% of that swept in the first run) in both cases, whereas additional treatments with SDS gave no appreciable signals.

4. Discussion

AFM has been recently used by our group for different purposes. In a first report, we have used it for exploring the roughness (or smoothness) of a bare fused-silica surface [34]. Our data showed the remarkable smoothness of such a surface, in agreement with the production process. In a second report [16], we have obtained some preliminary data on the quality of a positive coating obtained with the Immobiline technology [36]. In a third communication, we applied AFM to the task of evaluating the quality of soft polymeric coatings, as epitomized by polyacrylamide deposition onto silica surfaces [37]. In the present report, AFM is seen to be a useful tool for probing deposition of macromolecules onto the silica surface also. Interestingly, while there has been plenty of indirect evidence suggesting protein adsorption onto the capillary wall, especially at pH values below the p*I*, direct proof of such phenomena has been lacking up to the present time. The only other report we could find in recent literature was a poster presentation at the HPCE meeting in Orlando,

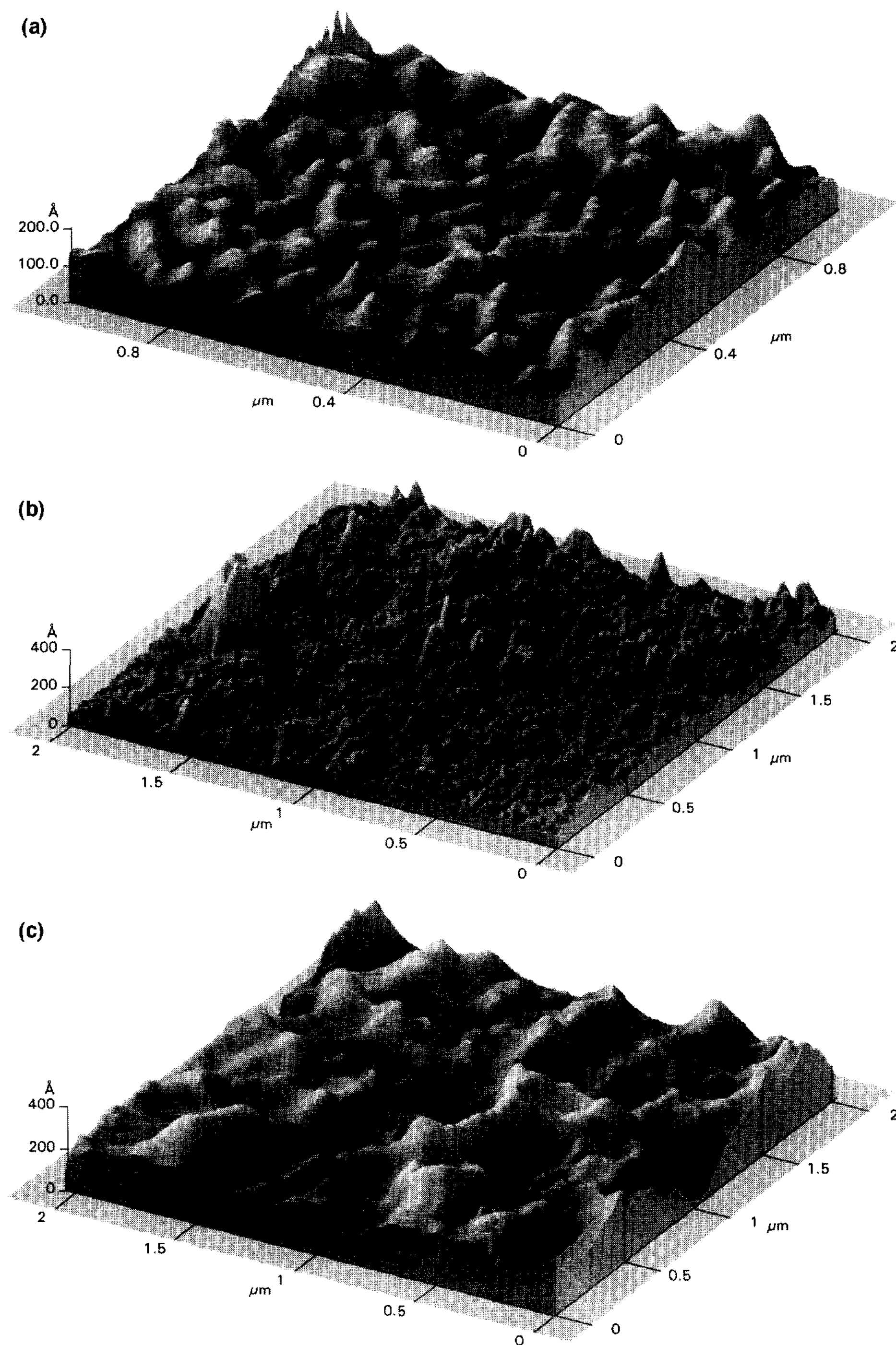

Fig. 5. Scan of a 2×2 μm surface area of the inner wall of a fused-silica capillary. (a) Control; (b) after adsorption of a 1.6 mg/ml ferritin solution at pH 7.0; (c) after adsorption of a 16 mg/ml ferritin solution at pH 7.0. Note, in (b), occasional peaks of proteinaceous material deposited on the surface. In (c), the silica wall is largely blanketed by a "vellum" of protein, reaching a thickness of about 40 nm.

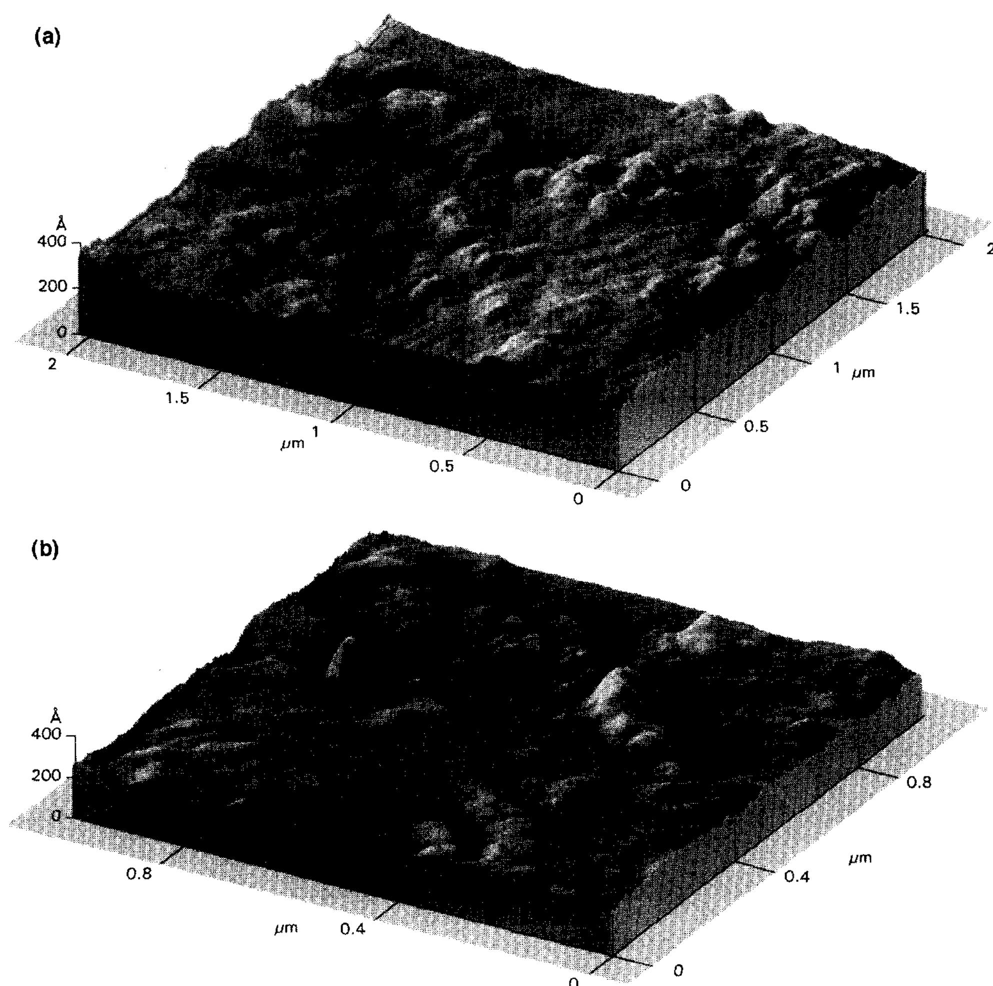

Fig. 6. Desorption from the silica surface after protein adsorption at pH 7.0. After incubation in 1 *M* NaOH of the same silica chips shown in Fig. 5b and Fig. 5c, the surface was probed again by AFM. (a) The original surface rugosity of plain silica, in the absence of protein, is restored (1.6 mg/ml load); (b) occasional peaks of ferritin, not completely rubbed off the surface, can be spotted (16 mg/ml initial load).

FL [38]. These authors use scanning electron microscope (SEM) both to probe the surface defects of a capillary and the potential adsorption of proteins to naked silica. In the first instance, their data are remarkably similar to our data obtained with AFM [34]: they find the depth of the surface defects corresponding to a 3 nm depth in the silica layer, in agreement with the average rugosity we have given of 4–5 nm. Additionally, they also find patchworks of deposited material (after running serum proteins in 60 m*M* borate buffer, pH 10) which they attribute to adsorbed proteins. Such proteins are not adsorbed as a continuous, homogeneous layer, but as clusters in different regions. The thickness of such adsorbed aggregates was estimated as >10 nm. There are striking similarities between our data and their data. We also find scattered clusters at pH>p*I* of ferritin (see Fig. 5b) but an almost continuous "vellum" both below and above the p*I* at high protein concentrations (16 mg/ml). Unfortunately, the protein concentration in their plasma samples is not given; in addition, since their primary aim was to analyse drugs in plasma, they have run sera containing SDS, which might have minimized protein adhesion to the wall. An additional, striking similarity, in both cases, is the shape of such deposits, which look very much

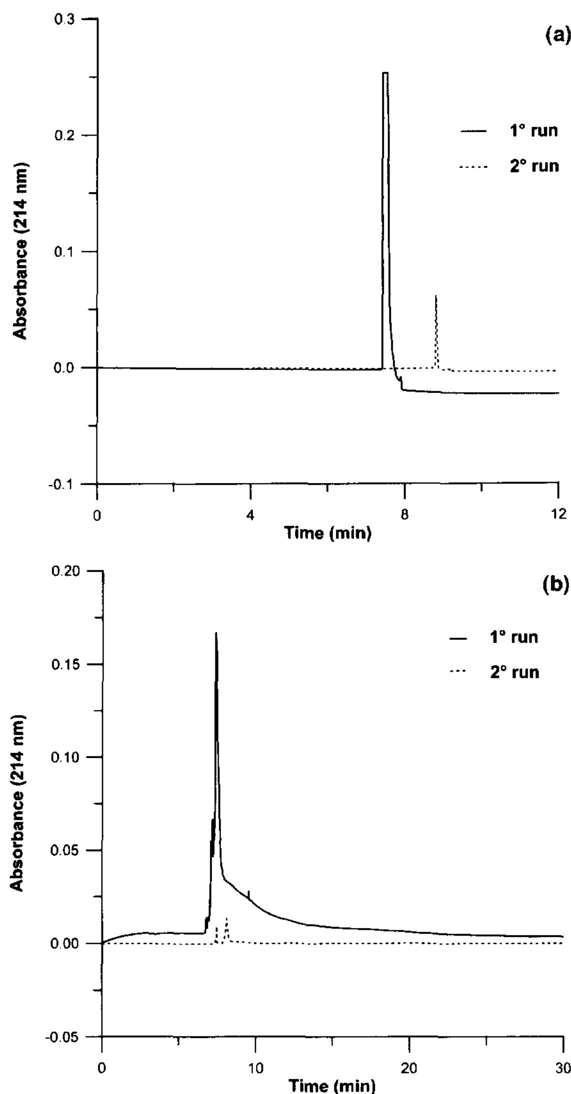

Fig. 7. Desorption of proteins adsorbed to the naked capillary by electrophoretic means. After ferritin adsorption, the capillary was subjected to electrophoresis either at pH 4.6 (panel a) or at pH 7.0 (panel b) by adding 30 m*M* SDS to the cathodic compartment. The appearance of a peak denotes frontal displacement of bound ferritin from the capillary wall. A small amount of protein (ca. 5%) was further released on a second run in both cases (broken lines).

like materials smeared out on the surface, rather than maintaining a characteristic globular shape of most proteins in solution. In fact, it might be asked why our AFM technique is unable to decipher the contour of ferritin, an icositetrahedral structure of globular shape, easily identifiable by SEM. One reason could be that the AFM tip and the object under analysis have almost identical sizes (10 nm for the former, 12 nm for the latter), which impedes a clear definition of the contours of the object. But we believe that the reason could be quite different: as the ferritin molecule adheres, by multi-point attachment, to the silica surface, it is probably distorted in shape and even denatured. Such molecules could therefore smear out and aggregate laterally to give this appearance of a continuous "vellum" deposited on the silica surface. In addition, judging from the thickness of the deposit, it is quite possible that in some regions the proteinaceous layer could be two to three molecules thick.

Our data contradict a widely accepted notion that, in order to prevent protein adsorption, all that is needed is to run such macromolecules at an operative buffer pH value two pH units above the p*I*, where such analytes would have a sufficiently high net negative charge. Given the fact that charged regions might be polarized on a protein surface, with clusters of positive charges well removed from negative ones, clearly the pH of the background electrolyte cannot per se guarantee absence of protein binding to silica surfaces. We also plan to use AFM to investigate two other aspects of protein analysis by capillary zone electrophoresis: (a) whether the addition of diamines and zwitterions to the background electrolyte can really eliminate protein adsorption, as claimed by a vast body of literature (see introduction); (b) whether even-coated capillaries are immune from protein adsorption. This last aspect is very important and is, at present, the subject of a vast debate. For instance, by isoelectric focusing of hemoglobin in well-coated capillaries (with a layer of polyacrylamide), we still find (by indirect evidence) binding of macromolecules to such coatings. This binding is much reduced (but, it would appear, not quite abolished) by coatings with more hydrophilic material, such as *N*-acryloyl amino propanol [39]. AFM technology could thus prove a very useful diagnostic tool capable of guiding chemists in designing better coatings, immune from protein adsorption, for the silica wall.

Acknowledgments

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References

[1] S. Hjertén, Chromatogr. Rev., 9 (1967) 122–219.
[2] S. Hjertén, J. Chromatogr., 347 (1985) 191–198.
[3] J.K. Towns and F.E. Regnier, Anal. Chem., 64 (1992) 2473–2478.
[4] F.E. Regnier and D. Wu, in N.A. Guzman (Editor) Capillary Electrophoresis Technology, Marcel Dekker, New York, 1993, pp. 287–309.
[5] S.V. Ermakov, M.Yu. Zhukov, L. Capelli and P.G. Righetti, J. Chromatogr. A, 699 (1995) 297–313.
[6] S. Hjertén, Electrophoresis, 11 (1990) 665–690.
[7] E. Grushka, in F. Dondi and G. Guichon (Editors) Theoretical Advancement in Chromatography and Related Separation Techniques, Kluwer, Dordrecht, 1992, pp. 607–632.
[8] M.R. Schure and A.M. Lenhoff, Anal. Chem., 65 (1993) 3024–3037.
[9] M. Minarik, B. Gas, A. Rizzi and E. Kenndler, J. Cap. Elec., 2 (1995) 89–96.
[10] B. Gas, E. Kenndler, A. Rizzi and M. Stedry, Electrophoresis, 16 (1995) 958–967.
[11] M. Stedry, B. Gas and E. Kenndler, Electrophoresis, 16 (1995) 968–975.
[12] M.S. Bello, M. Yu. Zhukov and P.G. Righetti, J. Chromatogr. A., 693 (1995) 113–130.
[13] E. Grushka, in N. Catsimpoolas (Editor) Methods of Protein Separation, Vol. I, Plenum Press, New York, 1975, pp. 161–192.
[14] T. Gu, Mathematical Modelling and Scale-up of Liquid Chromatography, Springer Verlag, 1995.
[15] M. Chiari, M. Nesi and P.G. Righetti, in P.G. Righetti (Editor) Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996, pp. 1–136.
[16] L. Capelli, S. Ermakov and P.G. Righetti, J. Biochem. Biophys. Methods, 32 (1996) 109–124.
[17] M. Chiari, M. Nesi and P.G. Righetti, Electrophoresis, 15 (1994) 616–622.
[18] J.L. Liao, J. Abramson and S. Hjertén, J. Cap. Elec., 2 (1995) 191–196.
[19] M. Bello, L. Capelli and P.G. Righetti, J. Chromatogr. A, 684 (1994) 311–320.
[20] H.H. Lauer and D. MacManigill, Anal. Chem., 58 (1986) 166–170.
[21] F.S. Stover, B.L. Haymore and J.R. McBeath, J. Chromatogr., 470 (1989) 241–250.
[22] V. Dolnik, J. Liu, J.F. Banks Jr., M.V. Novotny and P. Bocek, J. Chromatogr., 480 (1989) 321–330.
[23] J.A. Bullock and L.C. Yuan, J. Microcol. Sep., 3 (1990) 241–247.
[24] V. Rohlicek and Z. Deyl, J. Chromatogr., 494 (1989) 87–99.
[25] L. Song, Q. Ou and W. Yu, J. Chromatogr. A, 657 (1993) 175–183.
[26] N. Cohen and E. Grushka, J. Chromatogr. A, 678 (1994) 167–175.
[27] D. Corradini, A. Rhomberg and C. Corradini, J. Chromatogr. A, 661 (1994) 305–313.
[28] M. Zhu, R. Rodriguez, D. Hansen and T. Wher, J. Chromatogr., 516 (1990) 123–131.
[29] E. Watson and F. Yao, J. Chromatogr., 630 (1993) 442–446.
[30] P.K. Hansma, V.B. Elings, O. Marti, O. and C.E. Bracker, Science, 242 (1988) 209–216.
[31] J. Vesenka, C. Mosher, S. Schaus, L. Ambrosio and E. Henderson, BioTechniques, 19 (1995) 240–253.
[32] P. Arosio, T.G. Adelman and J.W. Drysdale, J. Biol. Chem., 253 (1978) 4451–4458.
[33] Cs. Horváth and W.R. Melander, in E. Heftmann (Editor) Chromatography, Part A: Fundamentals and Techniques, Elsevier, Amsterdam, 1983, pp. A27–A135.
[34] R. Barberi, M. Giocondo, R. Bartolino and P.G. Righetti, Electrophoresis, 16 (1995) 1445–1450.
[35] A.L. Shapiro, E. Vinuela and J.V. Maizel, Biochem. Biophys. Res. Commun., 28 (1976) 815–821.
[36] P.G. Righetti, Immobilized pH Gradients: Theory and Methodology, Elsevier, Amsterdam, 1990.
[37] R. Barberi, J.J. Bonvent, R. Rartolino, J. Roeraade, L. Capelli and P.G. Righetti, J. Chromatogr. B, 683 (1996) 3–13.
[38] S. Kaupp, R. Steffen and H. Wätzig, J. Chromatogr. A, 744 (1996) 93–101.
[39] M. Conti, C. Gelfi and P.G. Righetti, Electrophoresis, 1996, in press.