Probing soft polymeric coatings of a capillary by atomic force microscopy

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Abstract

Atomic force microscopy (AFM) has been used to probe the surface of a capillary after coating with "soft" polymers, notably polyacrylamides. The aim was the investigation of the efficiency of coverage of the silica surface, so as to reduce or eliminate the electroosmotic flow (EOF), particularly noxious in the separation of macromolecules. The quality of such coating is strongly dependent on two variables: temperature and pH. In the first case, progressively higher temperatures produce open silica patches, where no polymer seems to be bound. The transition from coated to largely uncoated surfaces occurs at 50°C. Also the pH of the polymerizing solution strongly affects the coating efficiency. Since in all coating procedures the monomer solution is not buffered, addition of accelerator (TEMED, N,N,N'N'-tetramethylethylendiamine) induces polymer growth at pH 10–11. These pH values generate hydrolysis of the siloxane bridge anchoring the bifunctional agent (Bind Silane, onto which the polymer chain should grow) to the wall. Thus, coating and de-coating occur simultaneously. Low temperatures during polymer growth (typically 10°C) and buffered solutions (pH 7, titrated after TEMED addition) ensure a most efficient and thorough coating, with virtual elimination of EOF: well coated capillaries exhibit residual EOF values, at pH 10, of the order of 10^{-7} cm^2 V^{-1} s^{-1} vs. a standard value for uncoated capillaries of the order of 10^{-4} cm^2 V^{-1} s^{-1}. The AFM data have been fully confirmed by direct measurement of EOF in coated and uncoated capillaries under an electric field.

Keywords: Polymer coatings; Atomic force microscopy; Polyacrylamide coatings

1. Introduction

Untreated fused-silica comprises a number of different acidic surface silanols, which impart to this surface, when bathed in any buffer above pH 2 under an electric field, some unique properties which dramatically affect separation efficiency in capillary zone electrophoresis (CZE). There are at least three types of ionizing groups: isolated, vicinal and geminal silanols. Interspersed among these ionogenic groups, one can envision inert siloxane bridges and highly acidic hydrogen bonding sites [1,2]. The total density of ionogenic silanols on this surface is given as $8.31 \cdot 10^{-6}$ M/m^2, corresponding to ca. five silanols per nm^2, with average pK values in the neighbourhood of 6.3 [3,4].

The electroosmotic force (EOF) in a capillary column is produced by an electric field and transmitted by the drag of ions acting in a thin sheath of

charged fluid adjacent to the silica wall column. The origin of charge in this sheath is an unbalance between positive and negative ions in the bulk solution which have to balance the fixed negative charge on the silica wall. Due to the fact that fused-silica capillaries today represent the standard electrophoretic cell in CZE, a number of studies have focused on ways and means of controlling EOF. One of the most disturbing phenomena in CZE is the unreproducibility of the EOF, particularly when subjecting the capillary to acid/base washing cycles (a standard procedure for cleansing the inner capillary surface after a series of runs). The hysteresis effect is sometimes so strong that it takes up to two weeks to restore the original EOF existing prior to such strong washing cycles [5].

While EOF per se is not dangerous in CZE, the mass of ionized negative charges bound to the wall is lethal in protein separations, since these macromolecules tend to bind cooperatively to ionized silanols and to be tenaciously adsorbed into the Debye–Hückel layer. An approach to solving this problem is to shield the silanol groups with a polymeric layer, either adsorbed or covalently bound. A large variety of polymers have been applied, such as linear polyacrylamide [6–8], cross-linked polyacrylamide [9], poly(vinyl alcohol) [10,11], epoxy polymer [12,13], polyether [14], cellulose acetate [15], polyethylene oxide–polypropylene oxide–polyethylene oxide triblock copolymers [16], polyethylene glycol [17], poly(N-acryloylaminoethoxy ethanol) [18], methyl cellulose [19] and polyacrylamide coated with zwitterionic surfactants [3-(dimethyldodecylammonio)propane sulfonate] [20]. Bonded hydrophobic phases have also been proposed: e.g., C_{18} coatings, either as such [21] or with adsorbed non-ionic surfactants [22]. A number of covalently bonded organic phases have additionally been described: trimethylsilane [23]; epoxydiols [24]; maltose [25] and arylpentafluoro [26,27]. Hjertén [6] is to be credited with some of the first reports on capillary coatings: in 1985, he proposed to covalently affix polyacrylamide "strings" to silica surfaces via a bifunctional agent, 3-methacryloxypropyl trimethoxy silane (commercially known as Bind Silane; in fact an agent already proposed by us in 1980 for linking macro-porous polyacrylamide slabs to glass surfaces) [28]. While this, as well as a number of other, "soft

coatings" have become quite popular in CZE for quenching and taming EOF, there are a number of questions still to be answered: the first regards the thoroughness of such coatings, i.e. the efficacy in covering the entire surface; the other regards the thickness of such coatings. A modern instrument which could offer some answers is the atomic force microscope (AFM).

The AFM is essentially a scanner that acquires topographical maps of a surface [29]. A very sharp tip 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 is ultimately related to the surface morphology. The experimental data thus obtained are fed into 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 at that point. In a way, the AFM measures topography by mechanically moving this sharp probe across the sample to "feel" the asperities of the surface much like a phonograph stylus was used to navigate the grooves of old vinyl records. The AFM has recently been shown to be a valuable tool in life science research [30]. In a recent report, we have first applied it to exploring the roughness (or smoothness) of a bare fused-silica surface [31]. Our data indeed showed the remarkable smoothness of such a surface, in agreement with the production process. In a second report [32], we have obtained some preliminary data on the quality of a positive coating obtained with the Immobiline technology [33]. In the present communication, we apply the AFM to the task of evaluating the quality of soft polymeric coatings, as epitomized by polyacrylamide deposition onto silica surfaces.

2. Experimental

2.1. Reagents

Acrylamide, N,N'-methylene bisacrylamide, tris (hydroxymethyl aminomethane), ammonium peroxodisulphate, and N,N,N'N'-tetramethylethylendiamine (TEMED) were obtained from Bio-Rad

(Hercules, CA, USA). Bind Silane [3-(trimethoxy-silyl) propylmethacrylate] was purchased from Aldrich (Steinheim, Germany).

2.2. Coating of capillaries

All capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA) and were of either 75 or 100 μm I.D. In all cases the outer diameter was 375 μm. They were treated first with 1% detergent, then with 1 M NaOH (10 min), rinsed with water, then with 1 M HCl (10 min), rinsed with water and dried in a oven at 120°C under a gentle stream of dry nitrogen. After reaction with Bind Silane, polyacrylamide strings were anchored to this surface by injecting a 4% acrylamide monomer (in the absence of cross linker) solution, degassed and added with persulphate and TEMED, under standard conditions [18]. The coating process was allowed to progress at temperatures ranging from 5°C to 80°C (overnight at low temperatures and 4 h at the higher temperatures). After coating, the excess viscous solution was pumped out and the capillary thoroughly rinsed with distilled water. The EOF flow was measured at various pH values (see relevant figures) by injecting 2 mM acrylamide as neutral marker.

2.3. Coating of silicium chips

Since it was quite difficult to tear open a coated capillary to explore its inner surface, the same treatments as in Section 2.1 were repeated on planar silicium chip surfaces, oxidized so as to produce a true silica surface, possessing the same silanol groups as a fused-silica capillary. The oxidation process was as follows: single crystal (<100> oriented) silicon wafers (thickness: 380 μm, diameter 9 cm, P type) were obtained from Wacker Chemitronic (Burghausen, Germany). The wafers were placed in a quartz tube, which in turn was inserted into a tubular furnace. The temperature of the furnace was 1100°C. Oxidation was performed by passing water-saturated oxygen gas (2 l/min, bubbled through water at 95°C) through the quartz tube for a period of 1.5 h. All procedures were carried out in a clean room. After processing, the wafers displayed a red colour, due to light diffraction. On the basis of this colour, the thickness of the

obtained silicon dioxide surface was estimated to be 0.8–0.9 μm.

2.4. Atomic force microscopy

The scanning probe microscope was from Park Scientific Instruments (Sunnyvale, CA, USA). A small capillary chip was mounted on the platform of the AFM 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 non-contact mode (also known as attractive mode, in which the AFM monitors attractive van der Waals forces between the tip and the sample). The data presented, though, are only those taken in the contact mode, since in this mode the surface is probed with higher resolution. Data are plotted on a 256×256 points digitizing grid. We have chosen three sizes of the scanned area: 0.5×0.5, 2×2 and 4×4 μm.

3. Results

The experiments evolved along two lines: in one, the quality of the soft polymeric coating was assessed with the AFM; in the other, the same coating was analyzed by direct EOF measurement in a capillary under an electric field. In order to facilitate data acquisition at the AFM, coating was performed on silicon wafers, after previous oxidation to silica, so as to obtain the same surface as in fused-silica capillaries. Wafers and capillaries were treated under identical conditions, except that, for the actual growth of polyacrylamide, polymerization was conducted in cassettes, as is customary in gel slab preparation.

Fig. 1 shows the quality of polyacrylamide coating (performed at 10°C) as explored on a 2×2 μm surface. The surface appears to be quite thoroughly and uniformly coated; very few "holes", if any, are apparent on the surface explored (note that the white areas represent protuberances, whereas the black circles should represents uncoated zones on the surface, possibly extending down to the silica wall). This is also confirmed by the three-dimensional plot of the scan depicted in Fig. 1a (Fig. 1b): it is seen that the surface is relatively smooth, with some
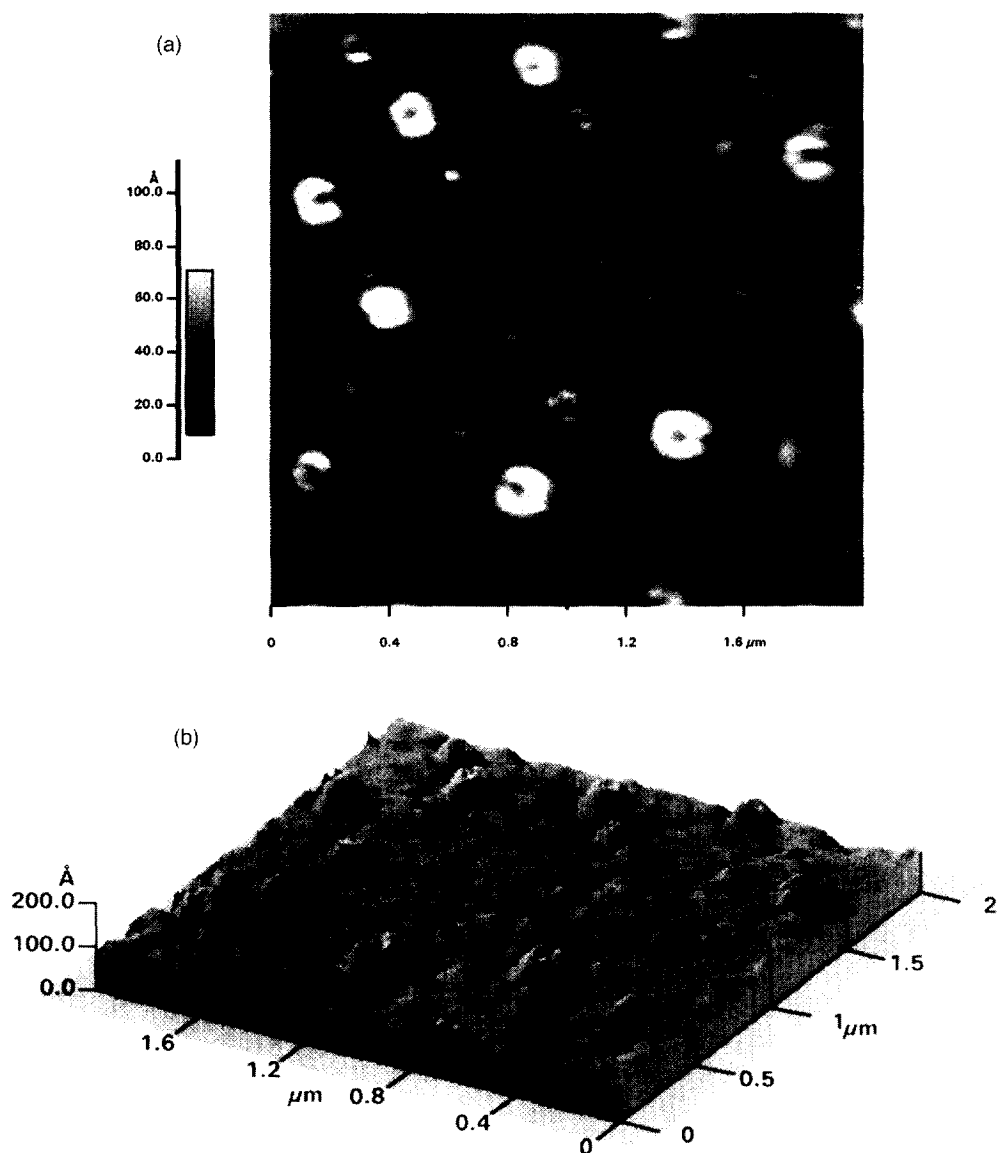
Fig. 1. (a) Scan of a 2×2 μm surface area of an oxidized silicon wafer coated with a covalently bound layer of polyacrylamide, as described in the text. Polymerization occurred at 10°C in non-buffered solutions (pH > 10). Note that the white areas represent peaks and dark areas valleys (probably reaching the floor, i.e. the bare wafer surface). (b) Three-dimensional representation of the surface area of the 2×2 μm scan of (a). Note that the z-axis is greatly expanded (10 nm total), as compared to the x- and y-axes, which cover a distance of 2000 nm. If drawn to the same scale, this surface "rugosity" should be reduced by a factor of ca. 100, thus it would appear completely smooth.

craters (probably representing condensed coating material) protruding and some pits embedded on it.

Fig. 2a shows the same polyacrylamide coating, this time performed at 20°C: now a series of black holes, which scan the surface at regular intervals, appear, suggesting non-uniformity of the coating.

This is also evident in the three-dimensional scan of Fig. 2a, in which some of the "holes" would seem to penetrate through the thickness of the coating.

Fig. 3a shows a scan of a 2×2 μm surface coated with polyacrylamide at 30°C. This time the "black" holes appear surrounded by a white rim. In the
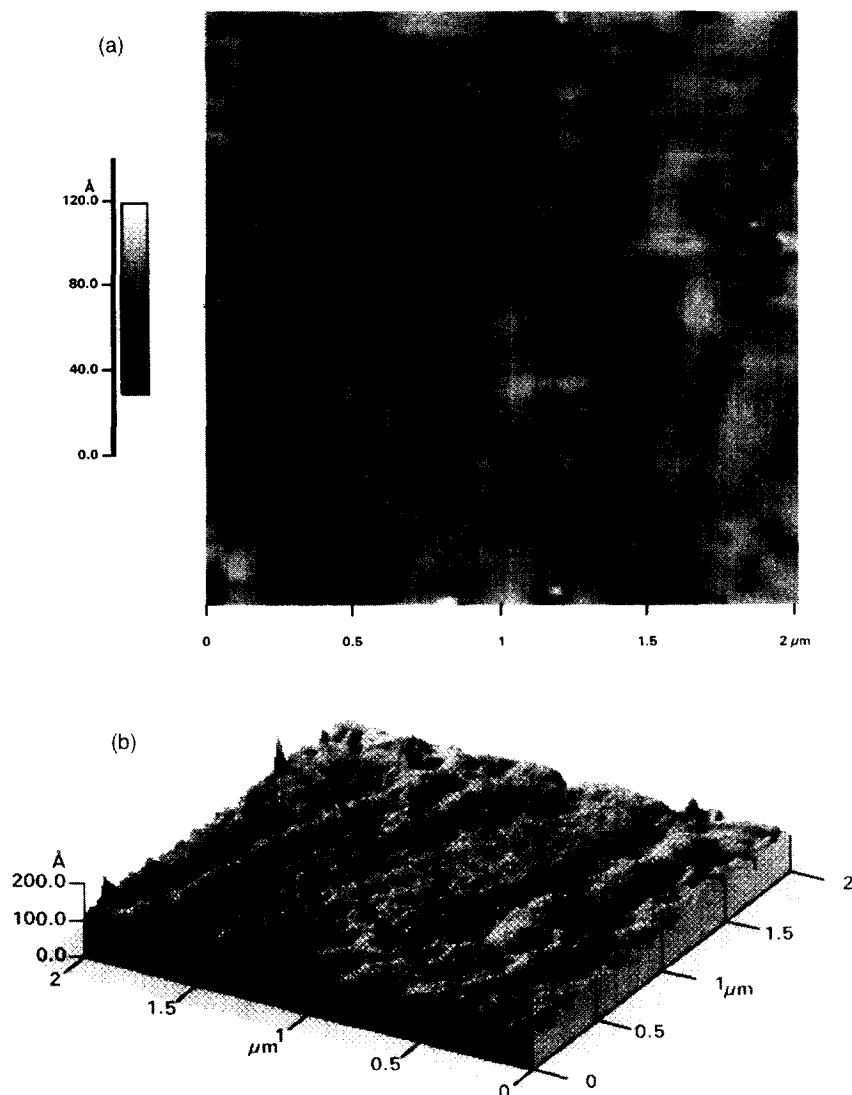
Fig. 2. (a) Scan of a 2×2 μm surface area of an oxidized silicon wafer coated with a covalently bound layer of polyacrylamide, at 20°C in non-buffered solutions (pH > 10). (b) Three-dimensional representation of the surface area of the 2×2 μm scan of (a). Note, in this case, the regular array of "holes" in both graphic representations.

three-dimensional scan of Fig. 3b, this is now seen as wide-mouth craters, protruding above the average 10 nm thickness by an additional 5 to 10 nm height. Thus, it would appear that coating at this temperature starts producing an irregular surface, with zones devoid of material and zones with condensed material.

In order to check whether this hypothesis is correct, we performed additional coatings at pro-gressively higher temperatures. As shown in Fig. 4 (a and b), deposition of polyacrylamide at 50°C results in a completely irregular surface, disseminated with pits and craters. From Fig. 4b, it is seen that most of these craters have a deep cone cutting through the soft polyacrylamide layer and reaching the floor (presumably the naked silica surface). This growth temperature of 50°C seems to correspond to a transition from a relatively regular surface, with

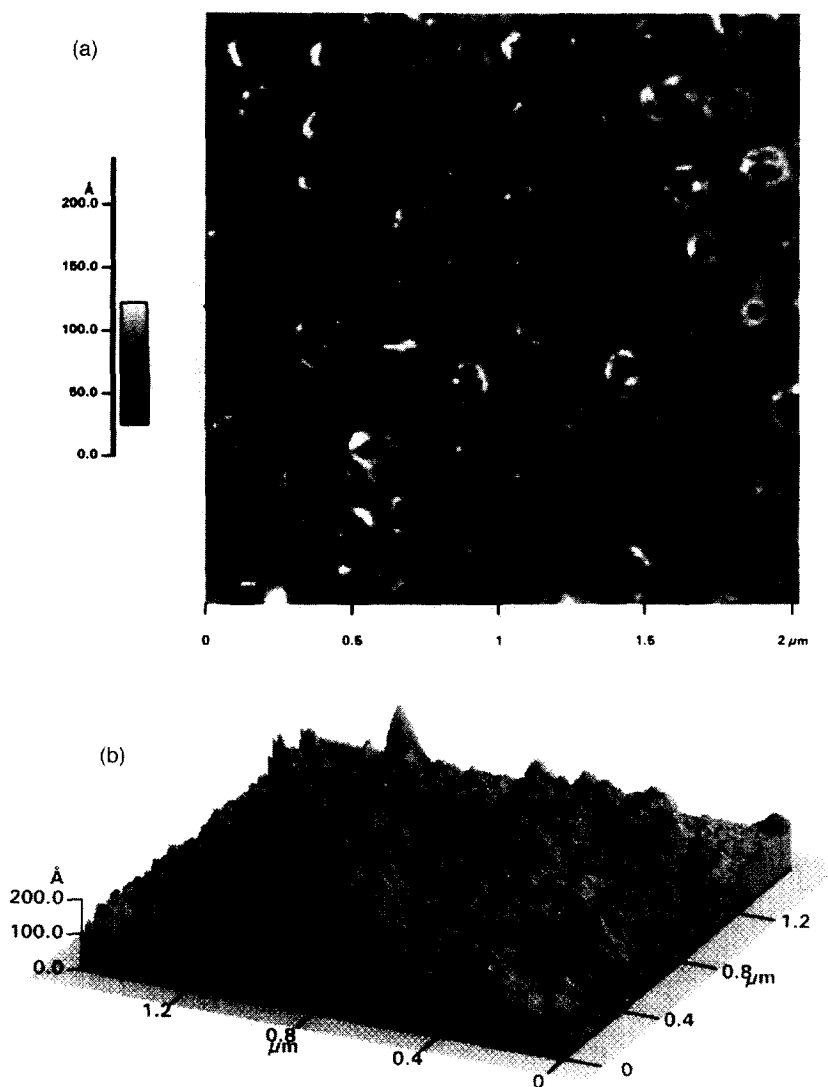Fig. 3. (a) Scan of a 2×2 µm surface area of an oxidized silicon wafer coated with a covalently bound layer of polyacrylamide, at 30°C in non-buffered solutions (pH > 10). (b) Three-dimensional representation of the surface area of the 2×2 µm scan of (a). Note, in this case, the scattered "holes" in the surface with corresponding white rims of condensed material, which look like "volcanoes" (with a mouth as wide as 100 nm) in the three-dimensional picture in panel (b).

scattered pits and craters, to a jagged surface showing only "volcanoes".

At even higher temperatures (70°C in Fig. 5 and 80°C in Fig. 6) the AFM images of the coating show a flatter surface with a high density of free spaces: the surface morphology now looks like a sponge and the uncoated areas (large black holes) seem to be connected by narrow, long channels. Thus, growth at

high temperatures (especially at 80°C) could be of dendritic type.

It might be asked how real these images are and whether or not we are seeing artefacts. The best way to confirm (or disclaim) the AFM data would be to measure EOF in a true capillary space coated by the same procedure. We have thus collected EOF data on different fused-silica capillaries, coated at 5°C and at

(a)

(b)

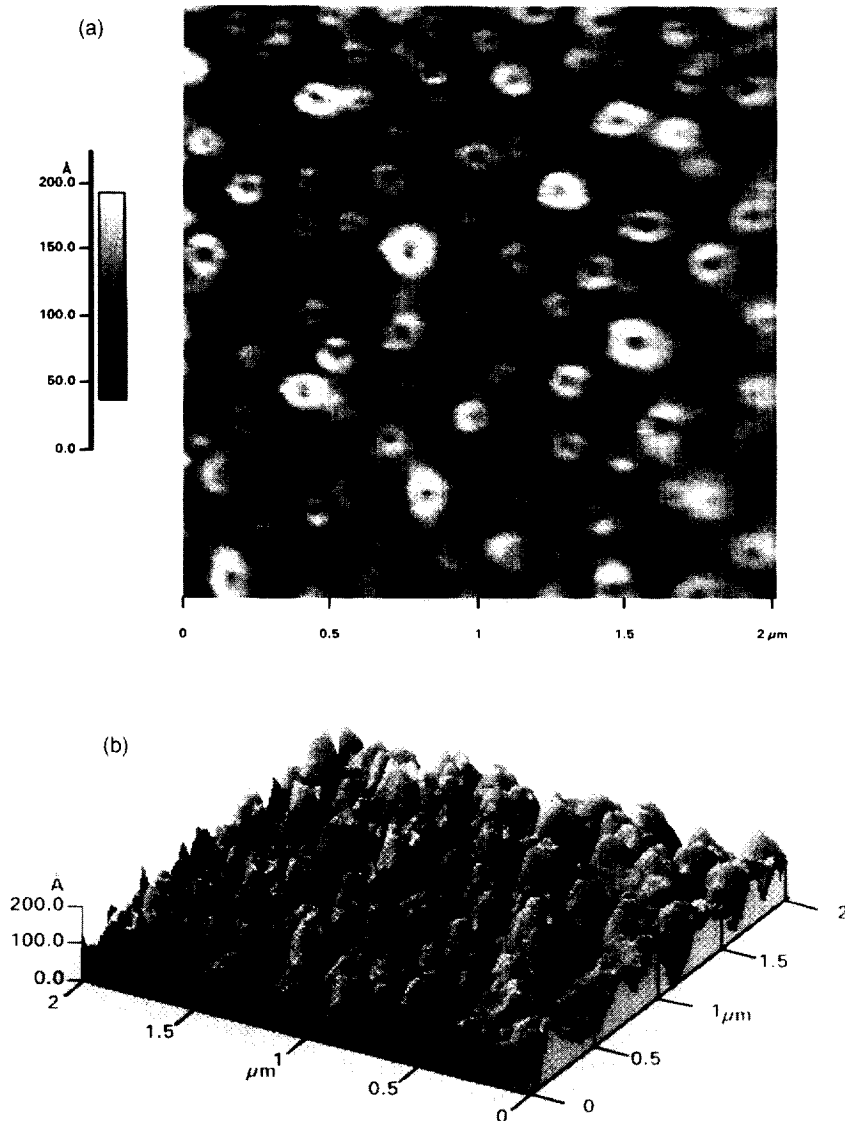
Fig. 4. (a) Scan of a 2×2 μm surface area of an oxidized silicon wafer coated with a covalently bound layer of polyacrylamide, at 50°C in non-buffered solutions (pH>10). (b) Three-dimensional representation of the surface area of the 2×2 μm scan of (a). Note, in this case, the transition from a relatively regular surface (Fig. 1 and Fig. 2) to a jagged surface scattered with pits and craters.

60°C. As shown in Fig. 7, the EOF measurements are dramatically different in the two surfaces, especially at alkaline pH (in Fig. 7 only the alkaline branch of the silanol titration curve is shown, as at progressively acidic pH values, data collection requires extremely long running times in the well-coated surface). It is seen that, at pH 10, the EOF value in a capillary coated at high temperatures is at

least ten times higher than the EOF exhibited by the same capillary coated at 5°C. Another parameter we have evaluated is the effect of pH on the coating. In all reports published so far, it would appear that the monomers are dissolved in pure water and then added with catalysts (persulphate and TEMED) just prior to the polymer growth process. Since TEMED is a free base, its addition would automatically
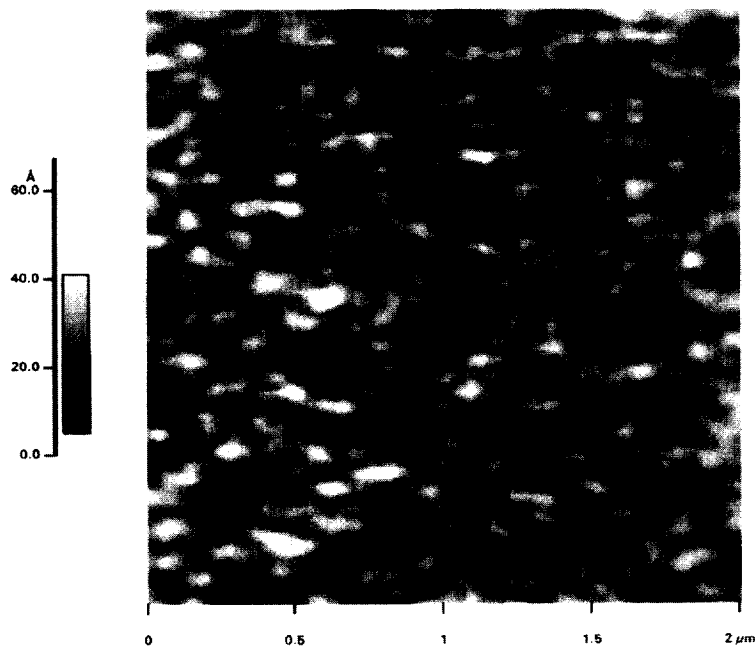
Fig. 5. Scan of a 2×2 μm surface area of an oxidized silicon wafer coated with a covalently bound layer of polyacrylamide, at 70°C in non-buffered solutions (pH > 10). The surface appears to have marked fractal characteristics.

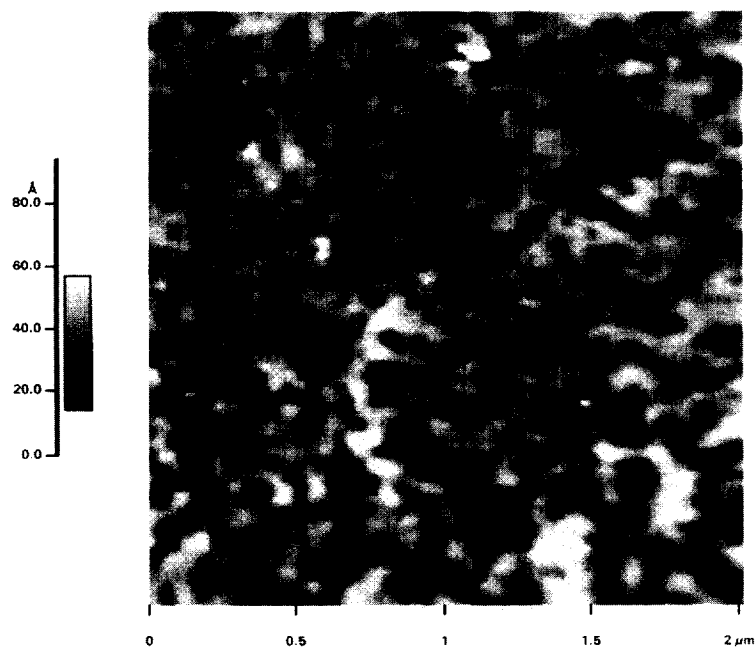Fig. 6. Scan of a 2×2 μm surface area of an oxidized silicon wafer coated with a covalently bound layer of polyacrylamide, at 80°C in non-buffered solutions (pH > 10). The surface appears to have a dendritic structure, with large holes (possibly representing naked silica surfaces) connected by narrow channels.
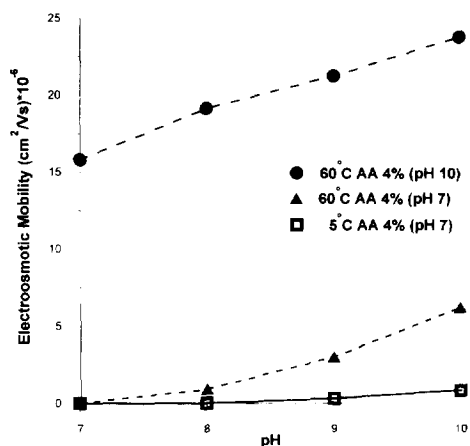
Fig. 7. Electroosmotic flow measurements in fused-silica capillaries coated by three different procedures: 5°C and 60°C in buffered (pH 7.0) solutions and 60°C in unbufferd (pH>10) solutions. Only the alkaline section of the silanol titration curve (pH 7 to 10) is shown. AA 4%: indicates the amount of monomer (4% acrylamide) used in the coating solution.

increase the pH of the gelling solution. The pH values measured in such unbuffered monomer solutions range, upon TEMED addition, from 10–11. We have thus prepared another series of capillaries, one coated in the absence of buffering ions, the other coated in a solution buffered at pH 7.0 (pH also adjusted after addition of TEMED). As shown in Fig. 7, under these conditions, the EOF in a capillary coated at high temperatures and pH 10 is at least 30 times higher than in one coated at low temperatures and pH 7. Thus the present data, obtained by direct control of both pH and temperature, appear to fully confirm the surface pictures displayed by the AFM technique, considering that in this last case, the coating was performed at progressively higher temperatures but without any pH control (i.e., coating occurred at the pH of the free base, pH 10–11).

4. Discussion

Our data begin to shed some light on some important parameters, often overlooked in surface coating chemistry. Two of them appear to be particularly important: temperature and pH, especially in regard to the growth of soft polymer coatings of the polyacrylamide type. We will analyze them separately below.

4.1. Effect of temperature on polymer growth

In the case of polyacrylamides, it is well known that temperature dramatically affects the length of the growing polymer. We have recently shown that, in presence of a chain transfer agent (2-propanol) and at high temperatures (typically 70°C), the length of a polyacrylamide chain can be considerably shortened, from >2 million Da (in the case of unperturbed growth) to ca. 200 000 Da in presence of these two effectors (the length of the chains referring to the weight average, $\bar{M}_w$, not number average) [34]. Clearly, the length of the chain anchored to the silica wall must have a profound effect on the EOF value. In fact, according to Hjertèn [6], suppression or elimination of EOF depends on the viscosity of the polymer layer bound to the wall. Short chain polymers have dramatically reduced viscosities compared to long chain polymers. Additionally, a very long chain polymer can, due to motion in the liquid layer, protect and "coat" (at least transiently, in time, if not in space) neighbouring uncoated surfaces, due to oscillation. Unfortunately, we have no way, at present, to assess the real thickness of the coating. Due to the type of AFM instrument we use, we can only probe dry surfaces (also by necessity, since water will immediately evaporate from the soft polymeric coating once exposed to air). Nevertheless, we can tentatively assess the thickness of the dry polymer layer to about 10 nm, as shown in the three-dimensional scans. If we assume that the fully hydrated polymer layer could gain a ten-fold excess of solvent, it is not unreasonable to assume that the thickness of the wet polymer layer should be at least 100 nm. How expanded or compressed this hydrated layer is as a function of temperature of polymerization is at present under investigation by ellipsometry.

There is, however, another important parameter, in addition to the actual length of the polymer bound to the wall, to take into account when investigating surface coating as a function of temperature. According to Nakatani et al. [35], temperature strongly affects the EOF value of coated capillaries: e.g., at 50°C, even well-coated capillaries rapidly degrade

and assume EOF values approaching those of uncoated capillaries. These authors have attributed these findings to instability of the siloxane bridge (a –Si–O–Si– bond linking the bifunctional agent, and thus the polymer, to the wall). Thus, the poor performance of capillaries coated at high temperatures could be attributed to two simultaneous phenomena: on the one hand, the growth of short polymer chains; on the other hand, the concomitant hydrolysis of the siloxane bridge linking the polymer to the wall.

4.2. Effect of pH on polymer growth

Surprisingly, it appears that no coating procedure has been described in which pH is controlled during the polymer growth process. However, by the same mechanism by which coatings are shown to be strongly sensitive to temperature [35], they appear to be even more sensitive to the pH of the buffering electrolyte bathing the wall. Two mechanisms can be advocated. At alkaline pH values, the siloxane bridge is rapidly hydrolyzed, thus detaching the polymer chains from the wall [35]. Additionally, as reported by us [36], conventional polyacrylamides rapidly hydrolyse at high pH values, forming polyacrylate: the negatively charged polymer chains will be forced to stretch and repulse due to Coulombic forces. In this extended configuration (as opposed to a collapsed state of neutral polymers) hydrolytic agents will have easier access to the wall and provoke additional hydrolysis of the polymer itself and of the binding agent. Under these conditions, quick deterioration of the polymer coating is to be expected. Thus, it is not surprising that polymer deposition at high temperatures and high pH values would result in the fractal polymer growth seen in Fig. 5 and Fig. 6. On the other hand, there are no a priori reasons why the coating process should not be performed at neutral pH. Caglio and Righetti [37] have in fact demonstrated that the efficiency of polymerization with the red/ox couple persulphate–TEMED is maximal at pH 10, but also quite efficient at pH 7. Persulphate polymerization is only fully inhibited at pH 4. Thus, growth of the polymer at neutral pH, and at low temperatures, if conducted for sufficient time periods (typically overnight), will have several beneficial aspects: (a), prevent or minimize hydrolysis of

siloxane bonds (the ones anchoring the polymer to the silica wall); (b), allow thorough and uniform coating and (c), permit growth of long chain polymers.

4.3. Effect of type of anchoring agent

Although not specifically evaluated here, data from several reports suggest that Bind Silane is certainly not the best bifunctional agent to ensure a firm anchoring of polymers to the silica wall. First, the bridge formed is of the siloxane type, i.e. quite prone to alkaline hydrolysis (it should be remembered that most electrophoretic protocols contemplate separations at alkaline pH values, typically >pH 8). Secondly, Bind Silane is a trivalent silicon derivative. Upon grafting to the wall, typically only one of the methoxy residues reacts with the wall silanols; the others are quickly hydrolyzed, thus generating additional free silanols bound to the bifunctional agent (see Fig. 1 in Liao et al. [38]). If these silanols cannot further react, they will automatically regenerate the EOF pump when adjacent to naked patches on the silica surface. Ultimately, novel types of anchoring agents, forming a direct Si–C link (much more resistant to hydrolytic agents) will have to be sought [39].

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