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J. R. Rasmusson
Linköping University

R. Erlandsson
Linköping University

W. R. Salaneck
Linköping University

M. Schott
University of Paris VII

D. T. Clark
SERC Daresbury Laboratory

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ADSORPTION OF FIBRINOGEN ON THIN ORIENTED POLY(TETRAFLUOROETHYLENE) (PTFE) FIBRES STUDIED BY SCANNING FORCE MICROSCOPY

J.R. Rasmusson[1,*], R. Erlandsson[1], W.R. Salaneck[1], M. Schott[2], D.T. Clark[3] and I. Lundström[1]

[1]Department of Physics and Measurements Technology, Linköping University, S-581 83 Linköping, Sweden
[2]Groupe de Physique des Solides, University of Paris VII, Tour 23-2, Place Jussieu, 75251 Paris Cédex, France
[3]SERC Daresbury Laboratory, RUSTI, Daresbury, Warrington, Cheshire WA4 4AD, United Kingdom

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Abstract

We have investigated fibrinogen adsorption on ordered poly(tetrafluoroethylene), PTFE, fibres deposited on hydrophilic and hydrophobic silicon substrates. Fibrinogen molecules appear to adsorb with their long axis perpendicular to the fibre direction for PTFE fibres having widths of less than 100 nm. On these thin fibres, fibrinogen apparently forms close packed bands or clusters, consisting of small integer numbers of molecules arranged parallel to each other. On broader (> 100 nm) PTFE fibres, the fibrinogen forms two dimensional networks. The orientation of the molecules in these networks is random in the central flat part of the fibres but perpendicular to the fibre direction at the fibre edges. As a tentative explanation, we propose that the observed orientation may be linked to the radius of curvature of the fibre surface.

Key Words: Adsorption, atomic force microscopy (AFM), biomolecules, fibrinogen, orienting substrates, protein, poly(tetrafluoroethylene) (PTFE), scanning force microscopy (SFM), tapping mode.

*Address for correspondence:
Johan R. Rasmusson
Linköping University
Department of Physics, IFM
S-581 83 Linköping Sweden
Telephone number: 46-13-282475
FAX number: 46-13-137568

Introduction

Adsorption of protein molecules on solid surfaces is involved in many phenomena of practical and theoretical interest. It is involved, e.g., in fouling in the food industry, in the interaction between implants and living tissue, and in surface oriented diagnostic methods. The adsorption of a protein to a solid surface is a complex process influenced by the properties of the protein as well as the substrate surface. In the past, most of the interest has been focused on how chemical properties and microstructure of the surface affect this interaction. Different microscopical techniques have been used to obtain images of adsorbed protein molecules. The new scanning probe techniques have made it possible to obtain images without any special sample preparation [1, 2], and, furthermore, to make high resolution surface structure observations not previously possible [12]. Geometrical orientation of macromolecules was demonstrated by Frommer *et al.* [9] who studied tobacco mosaic virus adsorption onto phase separated Langmuir-Blodgett films having islands of hydrocarbons in a fluorocarbon matrix. It was found that the macromolecules adsorbed on the fluorocarbon areas, but with a tendency to follow the edges of the hydrocarbon islands (which were 1.5 nm higher than the fluorocarbon part).

We believe it is also of interest to study protein adsorption on ordered surface structures with dimensions similar to the protein molecules themselves. In order to do this, we have fabricated highly oriented fibres of poly(tetrafluoroethylene), PTFE, on silicon substrates, with fibre widths in the interval 30-600 nm and heights in the 5-20 nm range. We found this type of surface very suitable for studying the influence of surface structure on protein adsorption. Furthermore, we argued that it would be interesting to study protein molecules with axial dimensions similar to the width of the smallest of fibres. For that purpose, we chose fibrinogen, a trinodular molecule ~45 nm long and ~6-8 nm in diameter.

We used scanning force microscopy, SFM [1, 22], to study surface topography with high vertical and spatial resolution. Earlier, we had studied thin PTFE films,

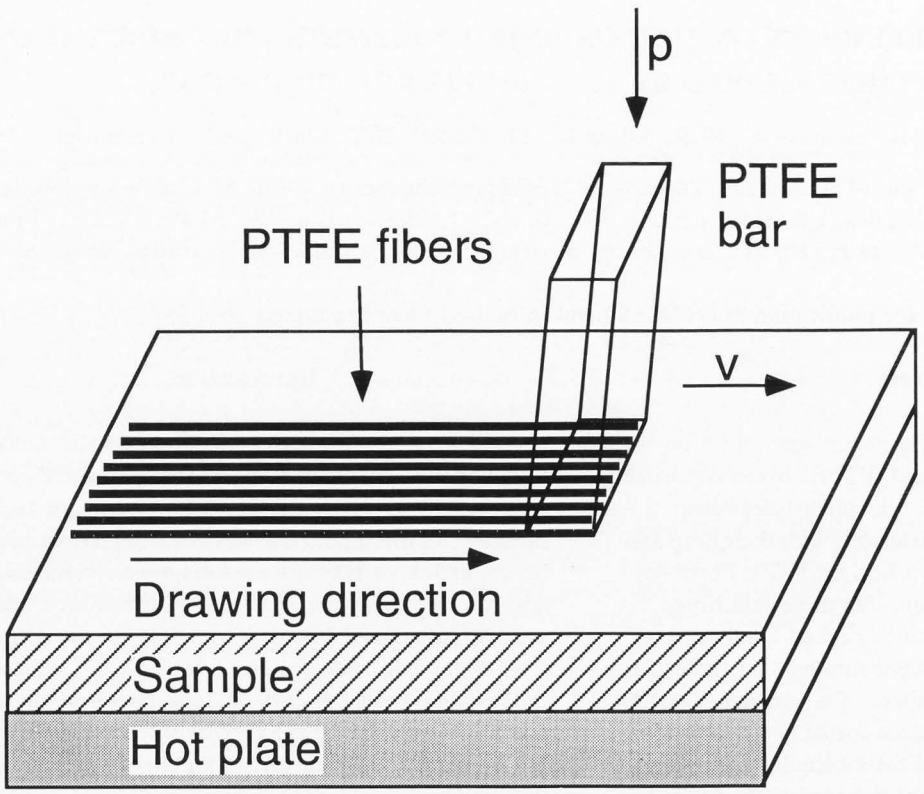

Figure 1. Schematic illustration of mechanical deposition of a thin layer of PTFE fibres on a solid smooth substrate, e.g., silicon. A solid bar of PTFE is dragged along the surface of the substrate at constant velocity, pressure and temperature.

initial adsorption of a conjugated polymer on PTFE, as well as fibrinogen adsorption on hydrophobic silicon surfaces [4, 8, 20]. The ordered PTFE structures were made following the method described by Wittman and Smith [21]. They found that a variety of materials, e.g., polymers, liquid crystals and small molecules, grew in a highly oriented form on PTFE. Their discovery appears to be promising for future device applications involving organic materials with controlled structure at the molecular level. The origin of the orienting effect of PTFE is not yet fully understood, but the surface topography of the PTFE films and a favourable crystal structure appear to be important factors. The film consists typically of fibres composed of parallel PTFE polymer chains all oriented along the fibre and with a periodicity of ~0.5 nm perpendicular to the chains [5, 11, 21]. In earlier scanning tunneling microscopy (STM) and SFM studies, we found that the degree of coverage, thickness, dimensions of PTFE fibres, etc., were strongly related to deposition temperature, pressure, velocity, and surface roughness of the substrate [4].

In this paper, we describe the preparation of the surfaces used and several findings related to the adsorption of fibrinogen on thin oriented PTFE fibres. Thus, the aim of this work was to determine if discrete and aligned hydrophobic PTFE fibres, deposited on smooth oxidised silicon substrates, can be used to impose ordering of adsorbed protein molecules with dimensions similar to the width of the fibres. It could provide a new approach to the study (or possibly tuning) of structural and functional features of proteins by using the tip in scanning probe techniques [3, 6, 19].

Materials and Methods

Substrates

10 mm x 10 mm pieces of polished silicon wafers (semiconductor device quality) with 1-2 nm native (spontaneously grown) oxide were used to prepare the substrates. To obtain an oxidised, hydrophilic silicon surface, the wafers were cleaned in a mixture of hot (80°C) H_2O-H_2O_2(30%)-NH_4(25%), (5:1:1 volume/volume, v/v), rinsed in milli-Q H_2O, cleaned in hot (80°C) H_2O-H_2O_2(25%)-HCl(37%), (6:1:1 v/v), and rinsed in milli-Q H_2O. Some silicon pieces were further treated and made hydrophobic by immersing them into 10% dichloro-dimethylsilane in trichloroethylene for 5 minutes followed by a rinse in trichloroethylene and ethanol. The silicon substrates used were either native or processed as described above to become hydrophobic or hydrophilic. The native silicon had properties very similar to that of the hydrophobic substrates.

Sample preparation

Thin (~5-20 nm thick) fibres of PTFE were prepared on the silicon substrates described above by the hot dragging method described by Wittman and Smith

[21]. As shown in Figure 1, a solid bar of PTFE was drawn along the substrate at constant velocity, pressure and temperature. This leaves a thin layer of highly oriented PTFE fibres on the substrate. The substrate temperature was ~200°C, sliding rate ~1 mm/s, with a pressure of ~10 N/cm^2. Typically, this only partially covers the silicon substrate with PTFE fibres (Figure 2a). It was thus often possible to compare the behaviour of fibrinogen on the amorphous oxide on the silicon surfaces with the behaviour on the hydrophobic PTFE fibres in the same SFM image.

Human fibrinogen, grade L (Kabi Vitrum, Stockholm, Sweden), was used. Fibrinogen is a blood protein and an important blood coagulation factor. It is a trinodular shaped molecule with a length of ~45 nm and a diameter of ~6-8 nm, consisting of ~3000 amino acids and having a molecular weight of ~340 kDa.

The fibrinogen was dissolved in phosphate buffered saline (PBS), pH 7.4, at concentrations of 0.1-1000 μg fibrinogen/ml PBS. Silicon substrates partially covered with PTFE fibres were incubated at room temperature in the fibrinogen/PBS solution for 5 minutes with slow agitation, rinsed in milli-Q H_2O, and dried in an N_2 gas flow.

Microscopy

SFM measurements were done using a Nanoscope III instrument in standard tapping mode [22]. In this imaging mode, the SFM tip-cantilever is oscillated at high frequency (~300 kHz) with a relatively high amplitude (~10-100 nm), so that the tip taps the surface once during each period. Major advantages compared to the more frequently used contact mode SFM are (a) considerable lower contact forces and (b) almost no shear forces. The risk of damaging the surface or dragging matter by the tip is thus reduced [6, 13, 22]. Our experience, when comparing the two modes imaging various organic molecules and polymers, has been that the tapping mode is preferred. All SFM measurements were performed in laboratory air at 20-25°C and at a relative humidity of 15-25%.

Results and Discussion

Figure 2a shows a SFM image of PTFE fibres on a hydrophobic silicon substrate with native oxide, viewed as a set of straight parallel objects throughout the image. As seen in the cross-section profile, the height of the PTFE fibres is ~5-6 nm which corresponds to ~10 layers of PTFE polymer chains. On other samples, we observe PTFE fibre heights in the range 5-20 nm, in good agreement with earlier studies [11]. The widths of the PTFE fibres used in our study are 30-600 nm. SFM measurements of the PTFE fibres in pure PBS buffer did not give rise to any changes in the appearance of the fibres. After rinsing and drying, no PBS could be detected on the PTFE fibres. Figures 2b-2d show the development of protein coverage of PTFE fibres on silicon substrates with increasing protein concentration. In Figure 2b, fibrinogen molecular clusters are seen as bands on the PTFE fibres. On thin PTFE fibres (< 100 nm), the bands appear perpendicular to the main direction of the fibres. On wider fibres, the bands form irregular structures indicating the start of network formation. The height of the bands is 6-8 nm. In Figure 2c, a more pronounced network of fibrinogen, height 6-8 nm, is formed on the widest PTFE fibres. In Figure 2d, the PTFE fibres are almost completely covered by a homogeneous layer of fibrinogen. The thickness of the fibrinogen layer is determined by height cross-section profiles in the "holes" (uncovered areas of PTFE fibres) seen in the image and is estimated to be 6-8 nm.

Figure 3 shows higher resolution images of fibrinogen covered PTFE fibres. The heights of the fibrinogen moieties, seen in the vertical cross-section in Figure 3, are less than 8 nm. This indicates that only one layer of fibrinogen is adsorbed on the PTFE fibres. The widths of the fibrinogen features, seen in the section of Figure 3, are all considerably smaller than the length of an unperturbed fibrinogen molecule (~45 nm) but, due to tip broadening effects, appear larger than the diameter of one unperturbed fibrinogen molecule (6-8 nm). This indicates that the fibrinogen molecules are oriented with their long axis perpendicular to the direction of PTFE fibres of diameters 100 nm or less. The measured widths of the fibrinogen clusters, as seen in Figures 3a and 3b, often appear to be given by low integer numbers of parallel fibrinogen molecules. The data is consistent with a 6-8 nm diameter of the molecules added with a tip broadening effect of the same order [16]. The broadening varied in different measurements, and, furthermore, the dispersion of the fibrinogen cluster sizes also varied. So far, it has not been possible to determine exactly the distribution of the cluster sizes. Methods for characterization of the tip shape and reconstruction of distorted probe microscope images due to finite size and imperfect shape of the tip [14, 15, 17] have not yet been included in our study. The left micrograph in Figure 3 shows the presence of a very thin fibre, ~30 nm in width, where the fibrinogen molecules appear to wrap around the fibre. Taking tip broadening effects into account, we find, however, that the narrowest features are close to the diameter of a fibrinogen molecule.

On broad PTFE fibres, the fibrinogen molecules form networks instead of clusters. At the edge of the wide PTFE fibres, however, where the fibres have their smallest radius of curvature, the long axis of the fibrinogen molecule lies perpendicular to the fibre. If two PTFE fibres are sufficiently close to each other, there is

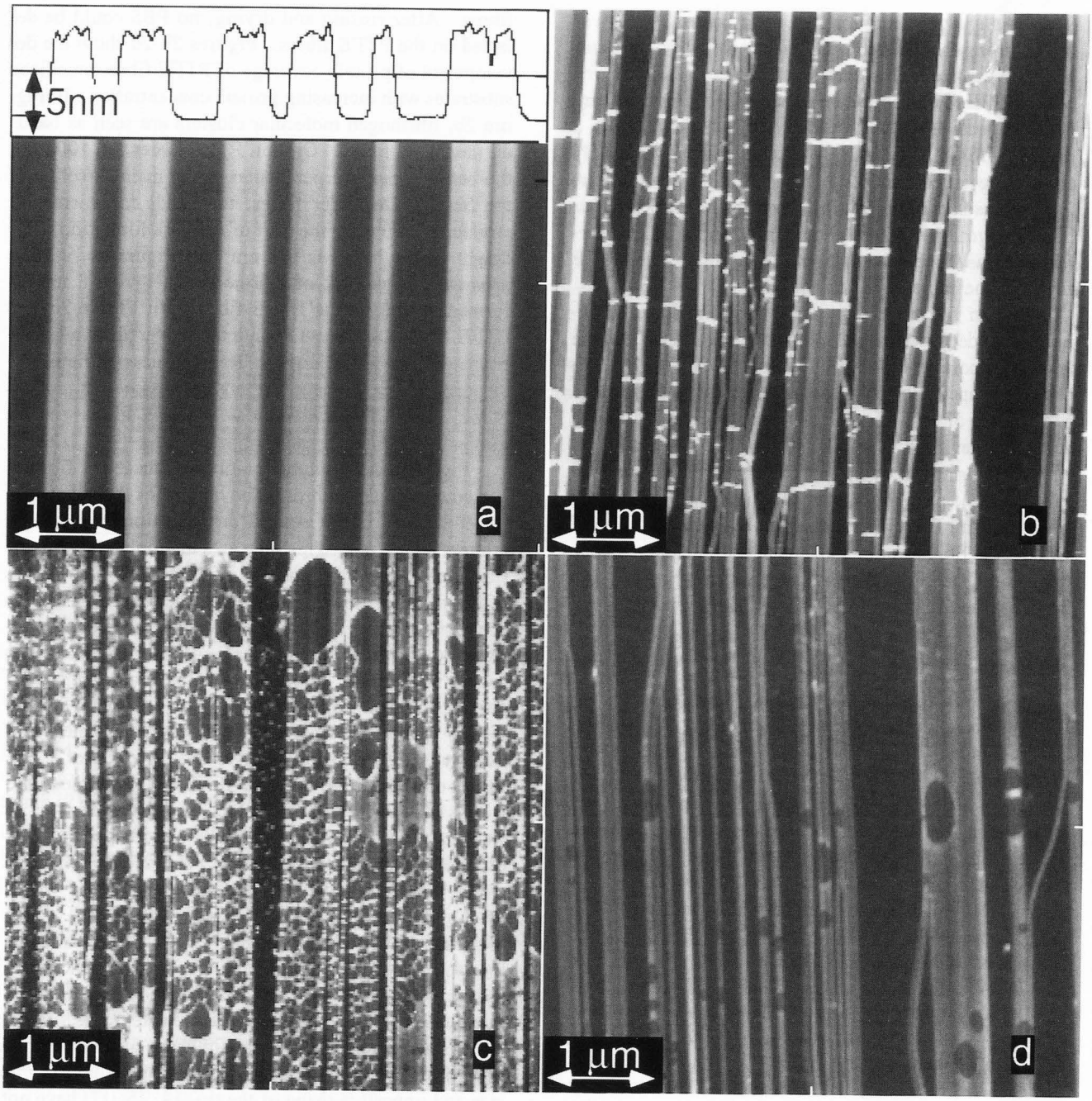

Figure 2. SFM images obtained using standard tapping mode in air showing the effects of different treatments. The contrast from black to white in all four images cover the 0-20 nm range. **a.** PTFE fibres deposited on native (spontaneously oxidised) silicon. The widths of the PTFE fibres are 30-600 nm. As seen in the cross-section profile, the height of the fibres was typically 5-6 nm corresponding to ~10 layers of PTFE polymer chains. **b.** 10 μg fibrinogen/ml on PTFE/native silicon substrates. Fibrinogen molecular clusters are seen as white streaks on the PTFE fibres. **c.** 100 μg fibrinogen/ml on PTFE/native silicon substrates. A network of fibrinogen is formed on the broadest PTFE fibres. **d.** 1000 μg fibrinogen/ml on PTFE/native silicon substrates. The PTFE fibres are almost completely covered by a thin layer of fibrinogen.

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a possibility of "bridging": a network of fibrinogen can be formed and bridge over the area between the PTFE fibres.

Cross-section profiles of SFM images indicate that this behaviour occurs only when the silicon in-between the fibres is covered by a thin layer of PTFE. The

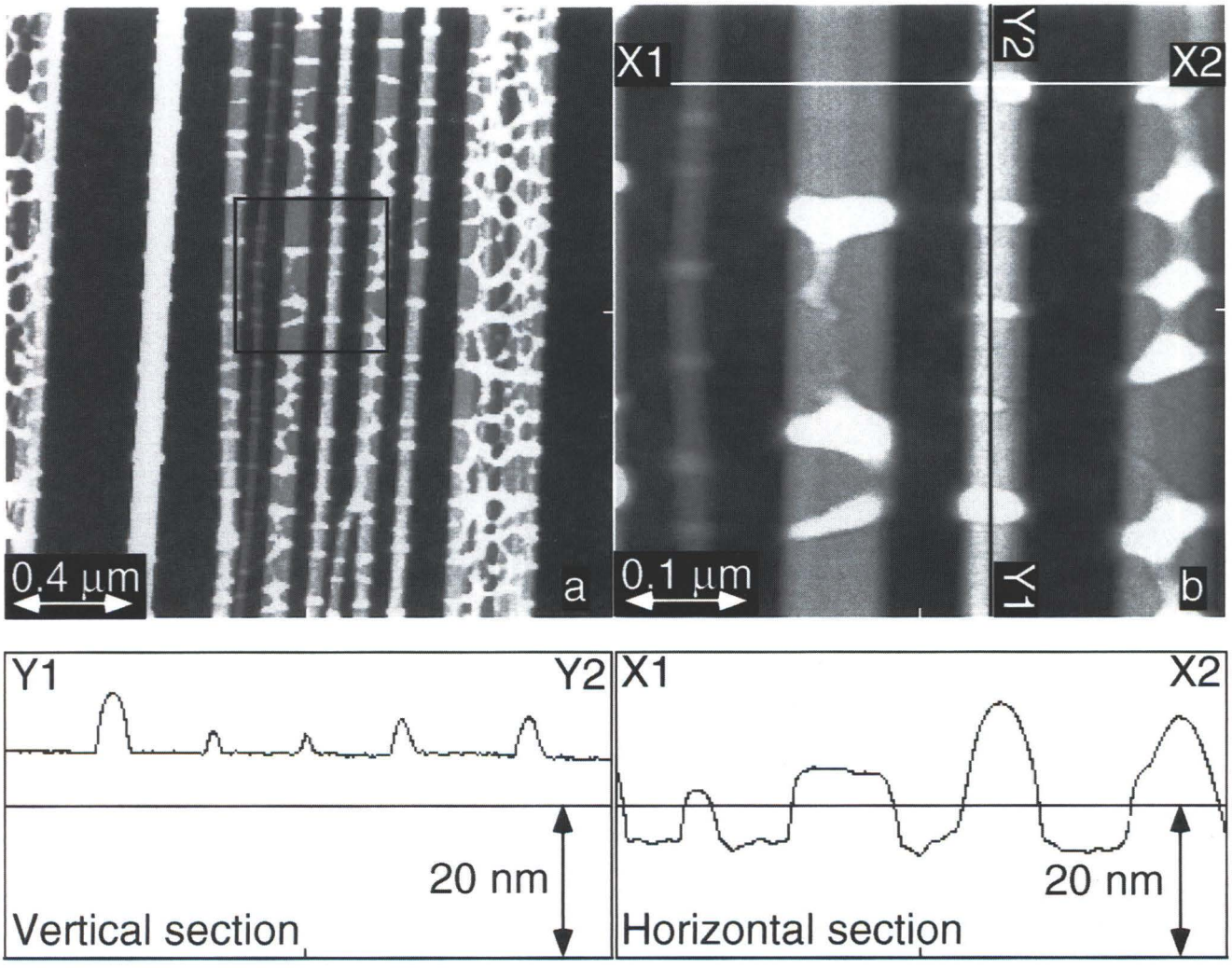

Figure 3. Views of the distribution of fibrinogen at an incubation concentration of 100 μg/ml, as well as cross-sections along a PTFE fibre and perpendicular to the PTFE fibres. Figure 3b is a magnification of the framed area in Figure 3a. The contrast from black to white in both images cover the 0-20 nm range. **a.** 100 μg fibrinogen/ml on a PTFE/native silicon substrate. **b.** 100 μg fibrinogen/ml on a PTFE/native silicon substrate.

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bridging effect is clearly seen to the right in Figure 3a where a fibrinogen network is formed between two adjacent PTFE fibres.

In Figure 4, images taken after the deposition of fibrinogen on PTFE/hydrophobic silicon substrates (Figs. 4a and 4b) and PTFE/hydrophilic silicon substrates (Figs. 4c and 4d) are shown. In Figure 4a, fibrinogen networks are seen between the PTFE fibres on the hydrophobic silicon substrates. This behaviour of fibrinogen on a hydrophobic surface has also been observed in previous studies [20]. These fibrinogen networks are spontaneously formed due to surface physical phenomena and thus differ from the fibrin networks observed on surfaces in solution, in the presence of fibrinogen and thrombin [7]. We observe that at the chosen magnification, the fibrinogen on the PTFE fibres looks like small pearls on a string. In Figure 4b, at higher concentration, the fibrinogen "pearls" on the PTFE fibres are more pronounced than in Figure 4a. The network between the PTFE fibres seen in Figure 4a is replaced by an almost uniform monolayer of fibrinogen. At some places, there are holes in the fibrinogen layer, especially close to the PTFE fibres. Height measurements at such holes indicate that the thickness of the fibrinogen layer

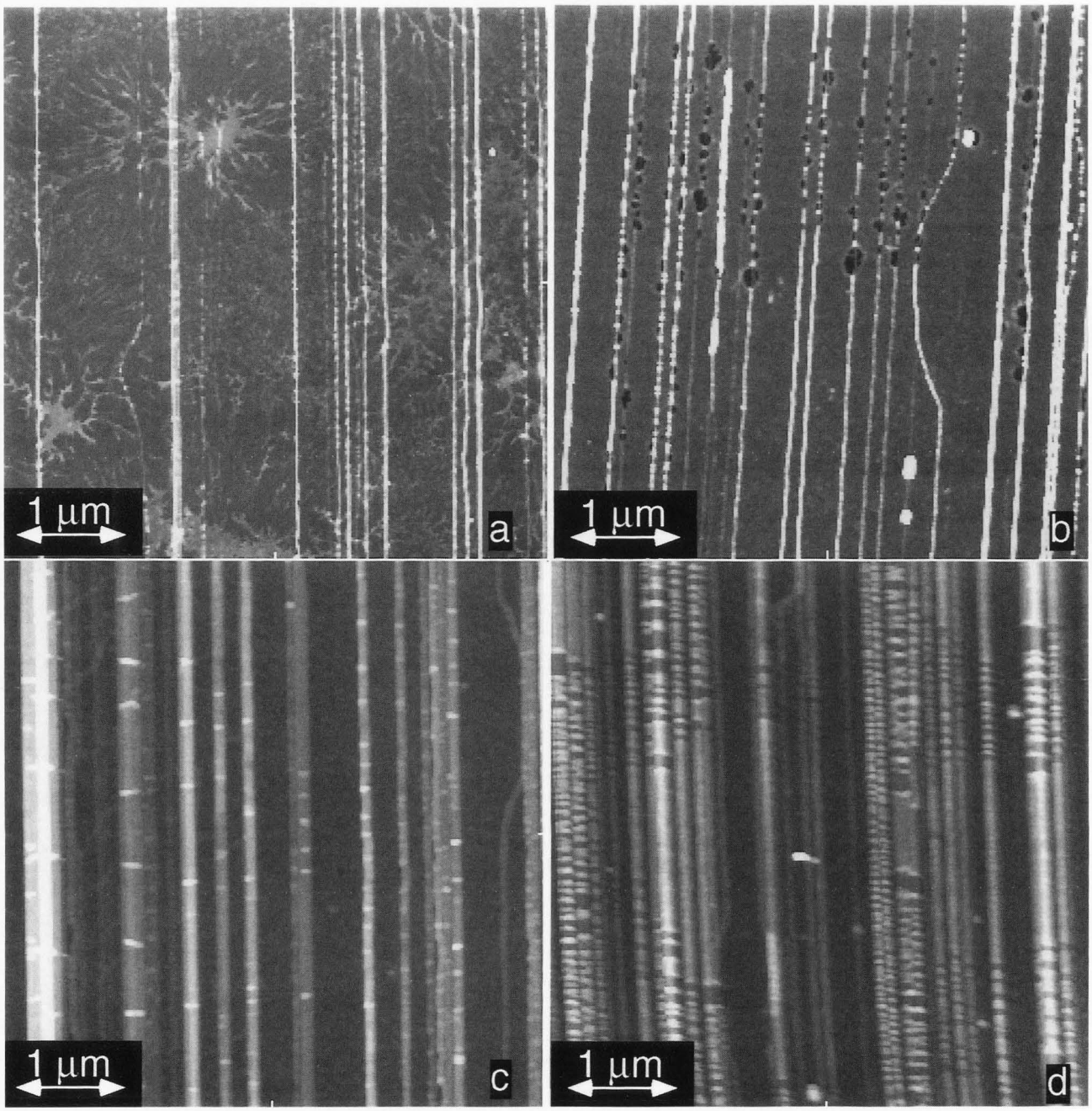

Figure 4. The figures show the distribution of fibrinogen on PTFE fibres deposited on two kinds of silicon substrates, hydrophobic and hydrophilic. The contrast from black to white in all four images cover the 0-20 nm range. **a.** 10 μg fibrinogen/ml on PTFE/hydrophobic silicon substrate. **b.** 200 μg fibrinogen/ml on PTFE/hydrophobic silicon substrate. **c.** 10 μg fibrinogen/ml on PTFE/hydrophilic silicon substrate. **d.** 200 μg fibrinogen/ml on PTFE/hydrophilic silicon substrate.

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is 6-8 nm, i.e., one monolayer. In Figures 4c and 4d, no fibrinogen networks are seen between the PTFE fibres. This is the typical behaviour of fibrinogen on hydrophilic surfaces. The fibrinogen "pearl" decoration of the PTFE fibres is again more pronounced at the higher concentrations (compare Fig. 4d with Fig. 4c).

The information in Figure 4 can be summarised as follows: the wettability of the silicon substrate influences only the fibrinogen deposition on the silicon surface exposed between the PTFE fibres. The behaviour of the fibrinogen on the PTFE fibres appears to be similar on hydrophobic and hydrophilic silicon substrates. This

indicates that the hydrophobicity of the substrate does not influence the pattern formation of fibrinogen on the PTFE fibres and that the areas between the PTFE fibres are unaffected by the PTFE deposition.

When protein macromolecules, like fibrinogen, adsorb on a hydrophobic surface, such as PTFE, they generally change their conformation to expose hydrophobic regions of the molecule to the hydrophobic surface. This will maximise the interaction between the surface and the molecule and is one of the driving forces for the adsorption to occur. Therefore, a possible explanation to the SFM results presented here is that when the radius of curvature of the fibre is small enough, it will be unfavourable for the molecule to adsorb along the edge as this will require an extra conformational change to obtain the same contact area. The molecule maximises its contact area with the fibre in these ways by a minimum change in conformation. The molecule will therefore adsorb perpendicular to the edge of broad fibres where the curvature is largest, while the orientation on the flat central part of the fibre will be random, (see Fig. 5). On thin fibres, both ends of the molecule (or a linear aggregate of molecules) can find a high curvature part of the fibre by orienting itself perpendicular to the fibre direction. Geometric tip broadening effects may make it difficult to resolve the phase boundary between the PTFE fibre and the silicon substrate accurately. It is unlikely, however, that for the thinnest fibres molecules adsorbed along the fibres would not have been detected, since the molecules have a diameter similar to these fibres. We therefore conclude that adsorption does not appear to occur at the phase boundary, i.e., along the side of the fibre.

As all measurements reported here were performed in air on dried samples, we do not know if the network formation and orientation of the molecules take place during the initial adsorption in the liquid phase or if it is induced during the drying procedure [18]. It has been observed that during drying of a hydrophobic surface with adsorbed protein molecules (IgG) the molecular distribution changes [18]. Patterns, similar to the fibrinogen networks in Figures 4a and 4b, and those observed earlier on hydrophobic silicon [18, 20], are found. It is therefore possible that a reorientation of the molecules on the PTFE fibres is driven by an optimization of the hydrophobic interaction between the molecule, the substrate and the surrounding air that takes place during the drying phase [18]. To determine at what stage the observed processes occurred, liquid phase experiments are needed which exclude the possibility of operating the microscope in the standard tapping mode, making it difficult to observe these fragile features. However, recently [10] a promising modification of the tapping mode has been developed that allows imaging in liquids. This new mode is similar to the standard tapping mode in air except that the sample is tapped against the tip instead of the cantilever being driven at resonance to tap the sample.

Our SFM images show that it is possible to align fibrinogen molecules on thin hydrophobic PTFE fibres. Fibrinogen molecules appear to adsorb, after rinsing and drying, with their long axis perpendicular to the direction of thin (diameters < 100 nm) hydrophobic PTFE fibres. On these thin fibres, fibrinogen appear to be able to form close packed bands, or clusters, consisting of small integer numbers of molecules arranged parallel to each other. On wide (> 100 nm) PTFE fibres, we observe in the central part that the fibrinogen molecules form networks. At the edges, where the radius of curvature is smaller, we observe ordered bands of fibrinogen similar to those on thin PTFE fibres. We propose that this orientational effect is due to the local curvature of the PTFE fibres and/or phenomena during drying. Yet to be studied is how other proteins adsorb on PTFE fibres, and protein adsorption on chemically modified PTFE fibres and on other polymer fibres.

Acknowledgements

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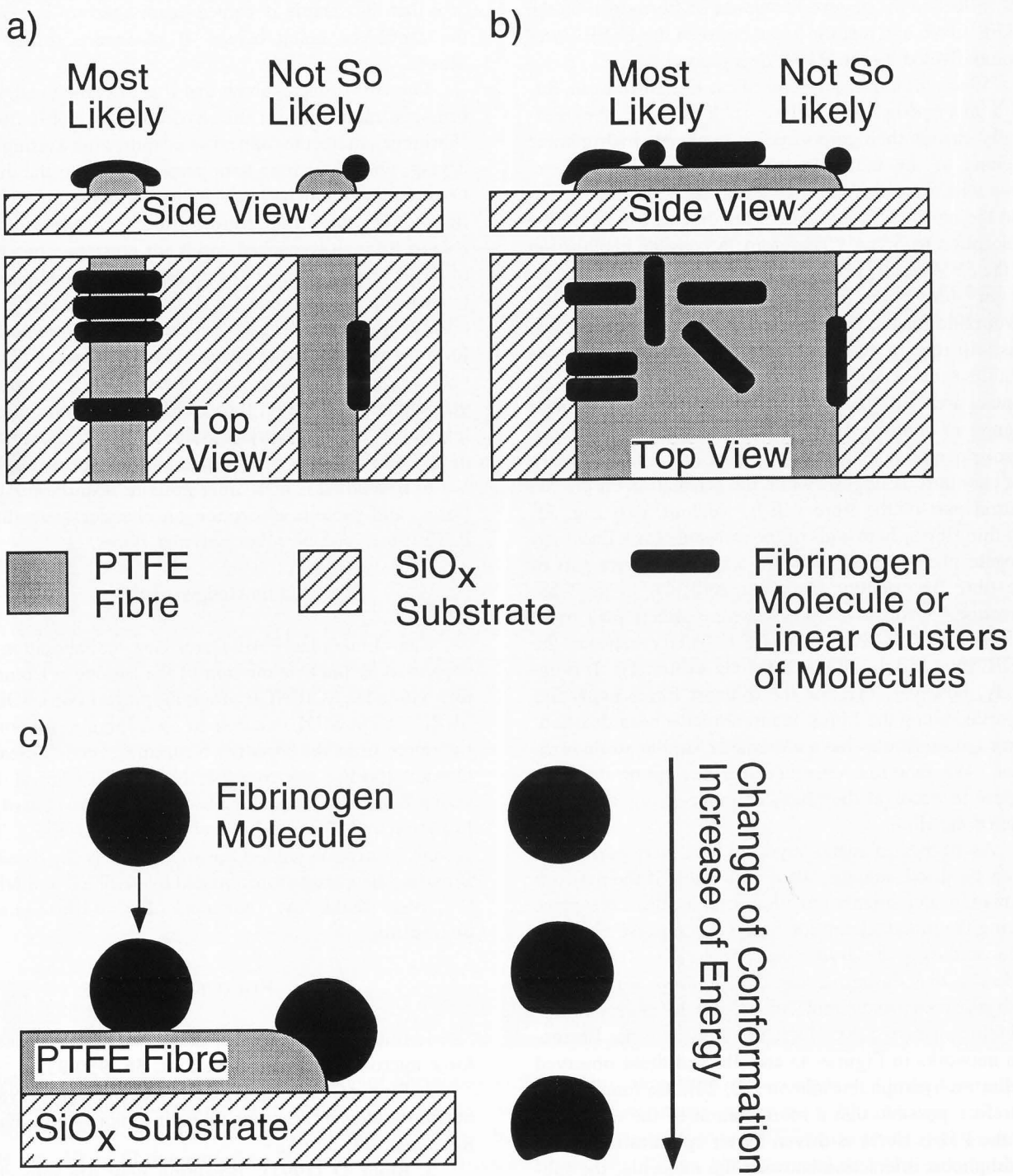

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Figure 5. **a.** The initial contact between an elongated protein molecule and a hydrophobic fibre with sufficiently small diameter is shown schematically. The protein is adsorbed with its length axis perpendicular to the fibre (left) or along the fibre (right) as viewed at a given cross-section of the molecule. The preferred orientation of a non-spherical protein molecule on a fibre with sufficiently small diameter is with its length axis perpendicular to the fibre direction because it optimises the contact between the fibre and the protein molecule. Even if the protein molecules are shown as monomers they may consist of several molecules linked together, all perpendicular to the fibre direction. **b.** A scenario is shown with a relatively broad fibre. In the central part, the fibre is smooth and has large radius of curvature so the difference between protein adsorption along and perpendicular to the fibre will be very small. Here, we expect to see a non-oriented or network forming fibrinogen distribution. Intuitively, this should occur when the fibre diameter is about 2-3 times that of the length of the protein molecule. However, at the edges of the fibre, where the radius of curvature is smaller, we expect the same orientational effect as in Figure 5a. **c.** A tentative "geometrical" contribution to the orientational effect: In the flat central parts of the PTFE fibre, less conformational change is required to obtain the same contact area between the fibre and the fibrinogen molecule than at the fibre edge. At the fibre edge, where the radius of curvature is small, the molecule will adsorb with its long axis perpendicular to the fibre. A parallel orientation requires larger change of conformation and is less likely.

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Discussions with Reviewers

J.L. Brash: Was the time of the adsorption varied?
Authors: All results shown are after 5 minutes adsorption. However, after 1 hour, a higher degree of adsorption was observed.

J.L. Brash: Was the time between the adsorption and the observations varied?
Authors: The time varied normally from a few minutes to several hours. No time effects were observed, not even after several days.

J.L. Brash: Is there, during adsorption, any possibility of a flow effect on orientation of the molecules?
Authors: This cannot be completely ruled out but different adsorption experiments varying the flow, rinsing and drying conditions gave similar results.

J.L. Brash: At the higher concentrations used in Figures 2 and 4, one would expect to be on or near the plateau of the adsorption isotherm with a surface concentration corresponding to a close-packed layer having at least partial end-on orientation. Can you comment on this aspect , i.e., the orientation in the flat part of the larger diameter fibres at relatively high concentration?

Authors: The observed height (6-8 nm) of the fibrinogen does not indicate end-on orientation of the fibrinogen molecules.

J.L. Brash: Have you looked at other proteins adsorbing on PTFE fibres?
Authors: Preliminary studies of BSA indicate monolayer adsorption (but different from that of fibrinogen) on the PTFE fibres.

H.G. Hansma: In the vertical section of Figure 3, why do you think the heights are so variable if they are all for one layer of fibrinogen?
Authors: The broad fibrinogen features are higher than the narrow ones. This could indicate that there is a larger conformational change and/or looser packing in the smaller clusters. Compression effects due to the tip load could also contribute.