Volume 280, number 2, 225–228 FEBS 09497 March 1991

ADONIS 0014579391002071

Structure of adsorbed fibrinogen obtained by scanning force microscopy

Roger Wigren, Hans Elwing, Ragnar Erlandsson, Stefan Welin and Ingemar Lundström

Department of Physics and Measurement Technology, Linköping University, S-581 83 Linköping, Sweden

Received 19 December 1990

It is shown that scanning force microscopy (SFM), operated in the attractive mode, can be used to obtain high resolution pictures of adsorbed fibrinogen molecules on solid surfaces, without the need for staining or special microscope grids. SFM also reveals the three-dimensional structure of the adsorbed molecules. Two forms of adsorbed fibrinogen are demonstrated on hydrophobic silicone dioxide surfaces: a trinodular about 60 nm long and a globular with about a 40 nm diameter. Polymeric networks formed after storage of the surface with adsorbed fibrinogen in PBS for 11 days are also shown. The SFM-results for the trinodular structure suggest the existence of loops or peptide chains extending outside the basic structure of the fibrinogen molecule.

Molecular structure; Fibrinogen; Adsorption; Scanning force microscopy; Atomic force microscopy; Polymerization

1. INTRODUCTION

Scanning force microscopy (SFM; also known as atomic force microscopy, AFM) is one of the new scanning probe techniques used to obtain topographic information about a surface with high resolution (nm or better) both vertically and laterally [1–3]. In SFM the force between a sharp probe tip and the surface is measured by attaching the tip to the end of a spring (lever) as is schematically shown in Fig. 1. The displacement of the lever is measured e.g. by laser interferometry [2,4,5]. Both short-range repulsive forces or the longer-range attractive interaction (used in this work) between tip and sample can be used. The scanning probe techniques are interesting in comparison with other microscopies like TEM and SEM. They can be performed in air and liquids [6] and do not require any special sample treatment. They yield a direct three-dimensional picture of surface structure or adsorbed molecules and do not need an electrically conducting sample. SFM has thus been used in aqueous buffer to observe the formation of fibrin polymers from fibrinogen and to make other dynamic in situ observations of proteins [6–8]. The details of the internal structure of the single molecules were, however, not revealed in these experiments. Fibrinogen molecules have also been imaged with STM (scanning tunneling microscopy) by other groups [9].

In the present paper we report studies of human fibrinogen adsorbed on a solid surface using a SFM operated in the attractive mode in air. Attractive mode measurements are of special interest in the study of biomolecules since they are more gentle to the molecules than the repulsive mode measurements normally used to obtain high resolution topographic pictures [10,11]. The tip scans closer to the surface in the repulsive mode than in the attractive mode and may subject molecules to forces (typically 10^{-9} N) which may alter observed structures [7,12]. It is therefore of considerable interest that we could obtain pictures of adsorbed fibrinogen molecules in the attractive mode, which apparently reveals more details than carefully performed TEM studies. The data we present here demonstrate how the SFM, operated in the attractive

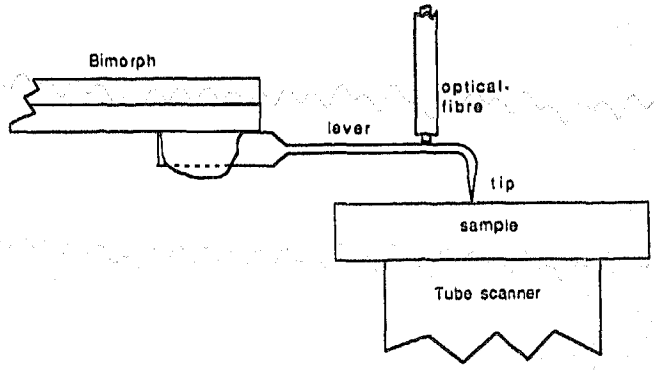

Fig. 1. A schematic illustration of the SFM. The tungsten wire is bent in a 90° angle and etched down to approximately 20 μm in diameter. In a second etching step, the end of the wire is formed to a sharp tip. The lever/tip is glued onto a piezo-electric bar (Bimorph) on which an electric AC-signal will induce the lever/tip to vibrate with its resonance frequency. An optical fibre guides the laser light (HeNe, 632.8 nm) to the lever. Interference between the reflected light from the fibre end and the lever is used to detect the lever motion. The sample is placed on a piezo-electric tube-scanner that enables motion in all three dimensions.

Correspondence address: R. Wigren, Department of Physics and Measurement Technology, Linköping University, S-581 83 Linköping, Sweden

mode, can resolve internal structure in fibrinogen adsorbed on hydrophobic silicondioxide.

2. METHODS AND MATERIALS

As substrates we used 5 × 10 mm² pieces of polished silicone wafers with native oxide (the same quality as used in the semiconductor industry) which first had been cleaned in a mixture of hot (80°C) $H_2O/H_2O_2/NH_4$ (5:1:1 v/v) rinsed in milli-Q water; then cleaned in hot (80°C) $H_2O/H_2O_2/HCl$ (6:1:1); and followed by extensive rinsing with milli-Q water. This treatment gives an oxidized and hydrophilic silicone surface. The Si-pieces were made hydrophobic by immersing them into 10% dichloro-dimethylsilane in trichloroethylene for 5 min followed by rinse in trichloroethylene and ethanol. The treated surfaces had a contact angle with water in the range 95–105° [13].

Fibrinogen was of Grade L obtained from KabiVitrum (Stockholm, Sweden) and was used without further purification. Fibrinogen molecules were adsorbed on the treated Si-surfaces by incubation at 1.0 μg/ml in PBS, pH 7.4, for 5 min or 0.2 μg/ml for 4 h at room temperature with slow stirring. The surfaces were then rinsed with milli-Q water and dried in a nitrogen gas flow.

The SFM-instrument used was designed and constructed in our laboratory [5]. It uses a fiberoptical laser interferometer as lever deflection sensor [4] and was operated in the attractive force regime by regulating the distance between the tip and the surface to give a constant frequency shift of the lever resonance induced by the force interaction [2,14]. In this mode of operation the tip will follow traces of constant force derivative over the sample. The liver/tip unit was made of an electrochemically etched tungsten wire with a right-angle bend and terminated in a sharp tip. The lever part was about 1 mm

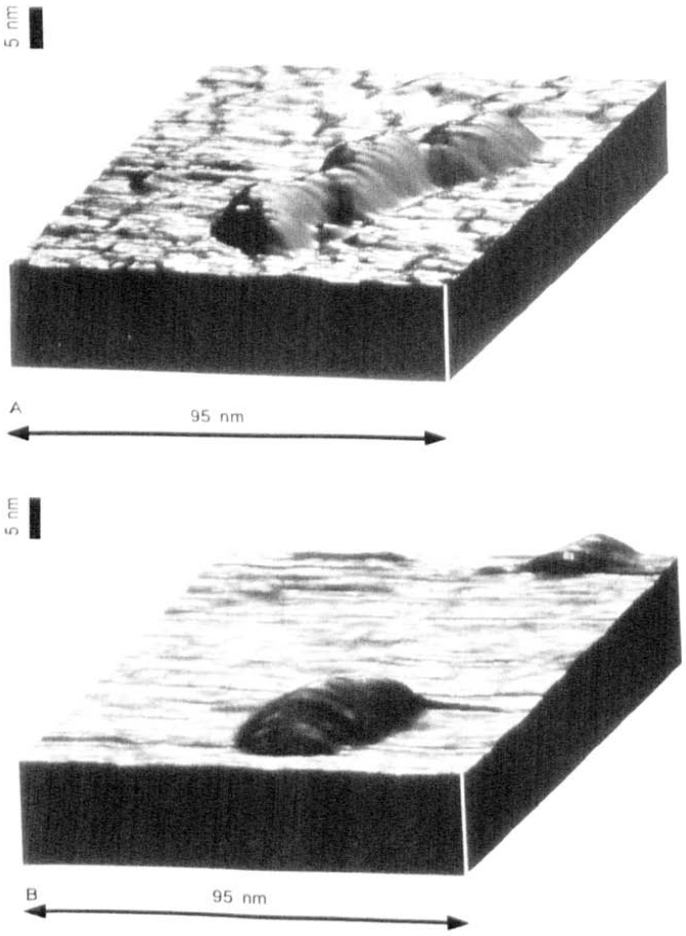

Fig. 2. 3D-rendered micrographs of the trinodular (A) and globular (B) forms of fibrinogen molecules on hydrophobic silicone dioxide surfaces (on two different samples). The scale bars denote the lateral scan size and the height scale, respectively. The silicone samples were immersed into PBS with 0.2 μg/ml fibrinogen for 4 h then rinsed in water and dried in flowing nitrogen gas. The surface concentration was less than 0.1 ng/mm² (the lower limit for ellipsometric detection) in both cases.

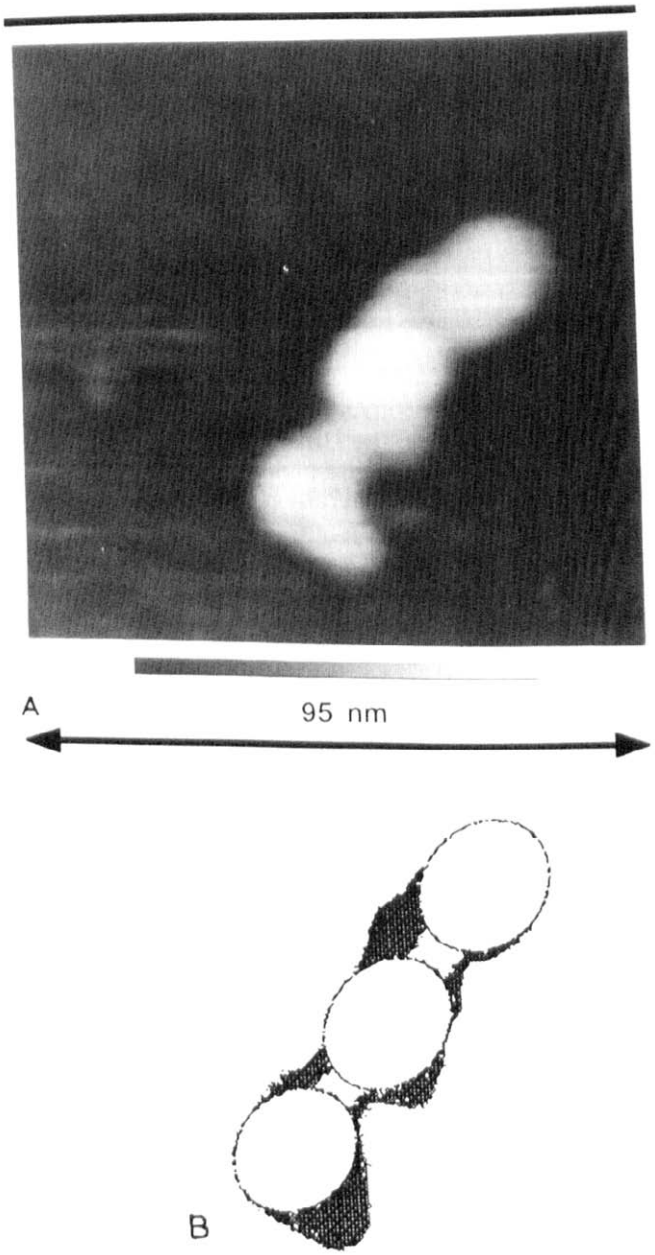

Fig. 3. (A) Top view of a trinodular fibrinogen molecule. The grey scale bar, corresponding to 6 nm, indicates the height scale. (B) Schematic structure of an (adsorbed) fibrinogen molecule. The dashed parts indicate extra projections, revealed by the SFM, that are surrounding the basic trinodular structure.

long with a diameter of approximately 20 μm, giving a resonance frequency in the 10–20 kHz range (different lever/tip units were used). The tip radii were typically 20 nm as viewed by SEM. In our SFM the lateral scanning is provided by a so-called piezo tube-scanner [15] on which the sample is placed. Each scan length was individually calibrated using optical interferometry to avoid errors due to the hysteresis effects in the piezo scanner.

The measurements were performed in laboratory air at room temperature.

3. RESULTS AND DISCUSSION

Fig. 2 shows 3D-rendered, topographical, pictures of two forms of fibrinogen molecules observed on hydrophobic silicone dioxide surfaces. One form is trinodular with a length of about 60 nm and the second has a more globular shape with a diameter of about 40 nm. The discrepancy from the generally accepted value of the length of the trinodular molecule (45–50 nm) is probably due to tip imaging effects [10,16]. The maximum height for both of the two forms is 6 nm. Fig. 2 illustrates well the type of information which may be obtained from SFM. The elongated trinodular, and the spherical forms with sizes of the same order as those found in Fig. 2 have also been suggested from recent TEM-studies on quartz (SiO_2) substrates [17]. The third dimension (the height) is, however, difficult to obtain from TEM. The elongated trinodular shape is a generally accepted schematic structure for the native fibrinogen molecule [18–22].

Fig. 3A shows a top view (i.e. the topography is presented as intensity variations of a grey scale) of a molecule in its trinodular form. In Fig. 3B the picture is compared to the schematic structure of fibrinogen discussed above. Tentative extra features as revealed by Fig. 3A are shadowed in Fig. 3B. Apparently the SFM-picture suggests some extra chains or loops outside the basic trinodular structure which are difficult to explain by tip imaging effects. The shape and extent of these extra structures varied somewhat between the different trinodular molecules, which could be expected for random-coil α-chains protruding from a randomly adsorbed molecule [23].

Fig. 4A shows polymeric structures found on hydrophobic surfaces which were stored in buffer after adsorption of fibrinogen for 11 days before the SFM measurement. The fibrinogen molecules form a network over the whole surface and the trinodular structure has disappeared (Fig. 4B). The width of one of the polymeric strands is about 12 nm. The individual branching points looked very similar and the take-off angles were almost always 120°, suggesting a symmetry in the polymer formation. This might be a consequence of a preferential orientation of interacting (perhaps three) molecules in combination with lateral mobility during network formation. The network observed by us could be the initial network for the much broader network observed by Rudee and Price [23,24].

The main purpose of this communication is to point out the type of information which can be obtained with the SFM operated in the attractive force regime on adsorbed protein molecules studied in air. We achieve a lateral resolution far better than the tip radius of about 20 nm which could indicate that the measured force is not a simple van der Waals interaction, but also includes other contributions, e.g. from the capillary interaction of a liquid phase bridging the tip-sample gap

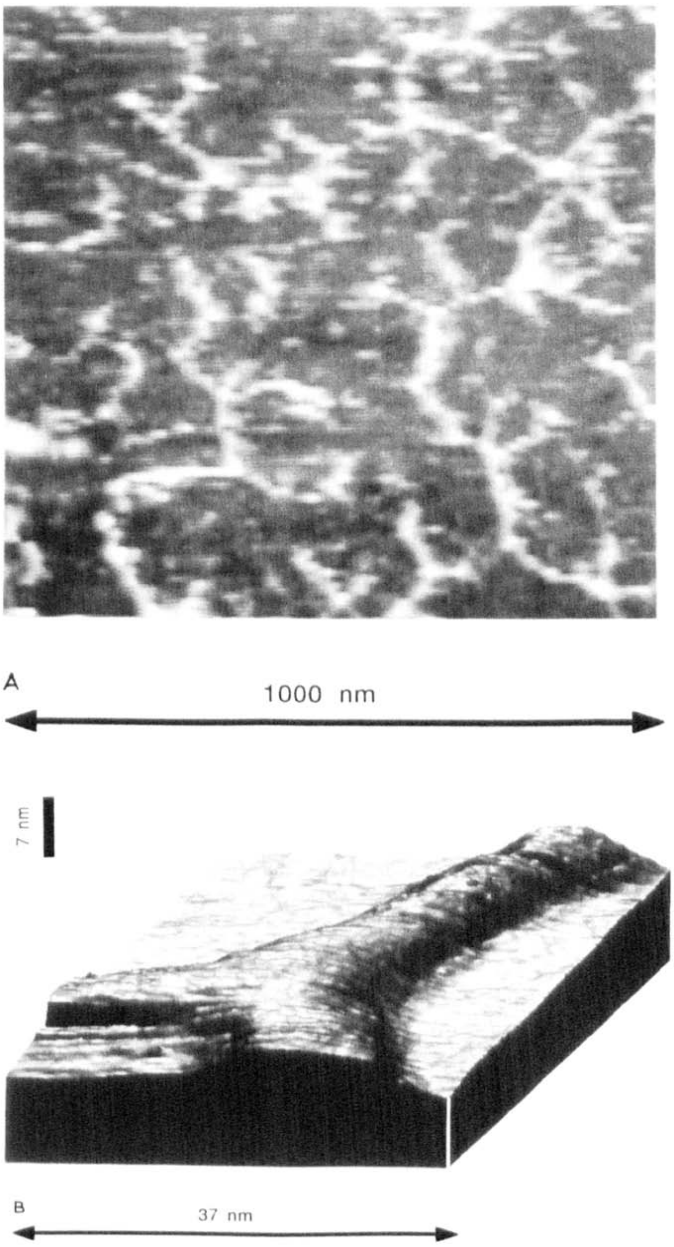

Fig. 4. (A) Top view of a polymeric network formed spontaneously on the hydrophobic surface after storage in PBS for 11 days. The silicon samples were immersed for 5 min in PBS with 1.0 μg/ml fibrinogen before the storage period. (B) 3D-view of a branching point. The scale bars denote the lateral scan size and the height scale, respectively.

[16]. We were able to image structures which are not directly visible in TEM, and obtain our data without any sample preparation that could disturb the system. We should remember, however, that the shape of the fibrin molecule in Fig. 3A may to a large part be due to the interaction with the surface. Further experiments will be made to elucidate the influence of such effects as the surface energy on the structure of the protein molecule. The globular shape occurs together with the trinodular form on the hydrophobic surfaces which may indicate molecular reconstruction after adsorption. At higher concentrations of fibrinogen other groups also report spherical fibrinogen using TEM [17] as well as molecular networks (M. Stenberg, personal communication).

There are several studies which can be suggested with the SFM. Since the preparation of samples is very simple it should be possible to perform a number of experiments related to the interaction between protein molecules and different types of surfaces. This should allow interactions such as those between proteins and their antibodies to be visualized by the SFM. Further studies should also provide new information on the effects of different staining techniques on molecules. The ability to obtain high resolution micrographs and to observe three-dimensional structure of soft biomolecules are qualities making the SFM, operated in the attractive force mode, a very promising technique for biology related measurements.

Acknowledgements: We thank Agneta Askendal for help with sample preparation, Lars Olsson for useful discussions and Peter Warkentin for kindly correcting this paper. This work has been supported by grants from the National Swedish Board for Technical Development (STU).

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