Fibrin Formation on Silicon Carbide – A Molecular Level Study with Scanning Force Microscopy

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Summary

The irreversible conversion of the plasma protein fibrinogen to fibrin is the key process in the coagulation cascade. In contrast to the plasmatic clotting pathway, the contact activation of blood is caused by an electron transfer from the fibrinogen molecule to the solid surface with direct contact to the blood. Thus, the structural and electronic properties of fibrinogen and artificial materials possess thrombogenic potential. Scanning force microscopy (also called atomic force microscopy, AFM) in the "tapping mode" is extremely useful for studying these processes. This method allows a structural analysis of biological objects and of solid surfaces at the nanometer level. In the presented study, the structure of fibrinogen was investigated at the molecular level. Its typical trinodular structure was revealed, which is approximately 52 nm in length. At higher concentrations, fibrin networks were imaged on silicon. Analysis of their molecular structure shows that the strands are built of fibrin molecules displaced parallel to each other. The correlation between contact activation potential and the electronic properties of fibrinogen and semiconducting materials was studied using AFM measurements. On silicon, the material with the smallest energy gap, fibrin was observed, whereas on the materials with higher energy gaps, only single fibrinogen molecules and small clusters of fibrinogen were present. The time-resolved imaging of the development of fibrin strands directly at the silicon-solution phase boundary was possible by AFM in the "contact mode". These results give further evidence of the correlation between electronic properties and thrombogenicity at a molecular level.

Key Words

Fibrinogen, fibrin, contact activation, hemocompatibility, scanning force microscopy

Introduction

A thorough understanding of the clotting processes that occur at the phase boundary formed between implants and blood is pivotal to the systematic development of hemocompatible surface coatings. The irreversible conversion of fibrinogen to fibrin is of particular importance for the process of contact activation. According to an electronic model describing the contact activation of the fibrinogen molecule, this process is induced by a charge transfer between the semiconducting protein fibrinogen and the surface of the solid [1]. Thus, the structural and electronic properties of the implant surface and the plasma constituents together determine the hemocompatibility of the device.

To investigate the fundamental statements of this model, the interaction between the fibrinogen molecule and solid surfaces was studied on a molecular level with scanning force microscopy (also commonly known as atomic force microscopy, AFM). In the "tapping mode", AFM has proven its value for the structural analysis of biological objects down to the submolecular level in the last few years [2]. The results taken from experiments at a molecular level contribute to the understanding of the protein-solid interaction and give new insights into the processes taking place during the contact activation of the protein fibrinogen at solid surfaces. Of special interest are: the molecular structures

of the fibrinogen and fibrin molecules; the molecular organization of the fibrin strands, which develop during contact activation; the time-resolved imaging of the development of fibrin strands; and the interdependence of the electronic properties of artificial materials and the fibrinogen molecule that leads to the development of fibrin strands at the implant surfaces.

Materials and Methods

Investigations were done with the Multimode-AFM, which was connected to the Nanoscope III controller from Digital Instruments GmbH. To minimize lateral forces and to allow for stable imaging of biological objects under air, the "tapping mode" was used [6]. In this mode the cantilever is oscillating at a relatively high frequency (100 to 500 kHz) as compared to the scanning frequency (about 1 Hz). Thus, the lateral forces during imaging are reduced by several orders of magnitude. Cantilevers were commercially available beam-shaped silicon nanoprobes.

Substrates were freshly cleaved mica and silicon wafers in the [111]-orientation. Additionally, hydrogen-rich, phosphorous-doped amorphous silicon carbide (a-SiC:H) was chosen as the prototype for a hemocompatible material [3]. All surfaces had a roughness of less than 0.5 nm, a prerequisite for the molecular resolution of fibrinogen and fibrin. The substrates were moistened for 5 minutes with a fibrinogen solution at 37 °C. The solution was prepared immediately before the moistening of the substrates by dissolving 1 to 6 µg/ml fibrinogen (Sigma, type IV from bovine plasma, 60% protein, 95% clottable) in physiologic saline solution. After incubation with the fibrinogen solution, the probes were rinsed with double-distilled water and air-dried.

Time-resolved imaging directly at the solid-solution phase boundary was done by AFM in the "contact mode" using a fluid cell. The lateral forces exerted on the molecules in this mode and in fluid are lower than the forces exerted in the "tapping mode" under air, because capillary forces are absent. For stable imaging during a period lasting up to 30 min, the concentration of fibrinogen was reduced by one order of magnitude to 0.4 µg/ml.

Results

On mica and silicon, reproducible imaging with submolecular resolution of single molecules was possible at low fibrinogen concentrations of 1 µg/ml. The image depicted in Figure 1 reveals the typical trinodular structure of the fibrinogen molecule. At higher concentrations, only small clusters of fibrinogen mole-

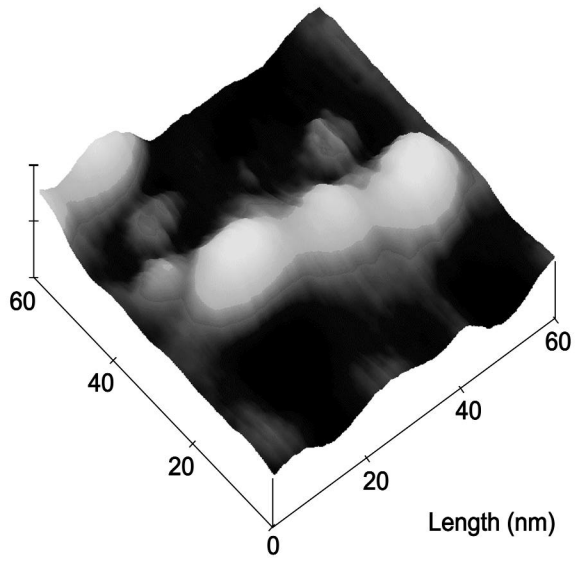

Figure 1. Isolated fibrinogen molecule imaged by AFM in the "tapping mode" on silicon (1 µg/ml fibrinogen).

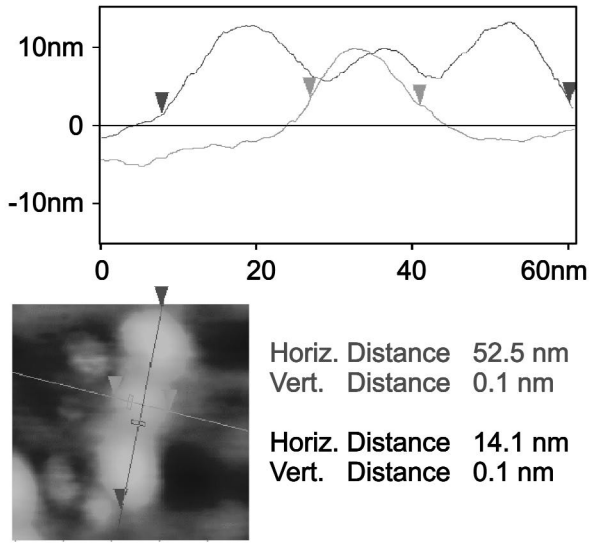

Figure 2. Isolated fibrinogen molecule imaged by AFM in the "tapping mode" on silicon (1 µg/ml fibrinogen) with the corresponding profiles of the protein.

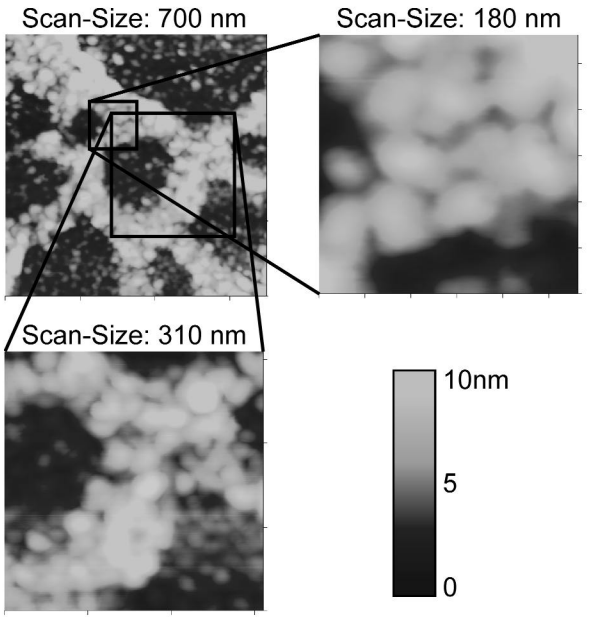

Figure 3. Fibrin network imaged by AFM in the "tapping mode" on silicon (4 µg/ml fibrinogen). In the two sections, single fibrin molecules are clearly resolved.

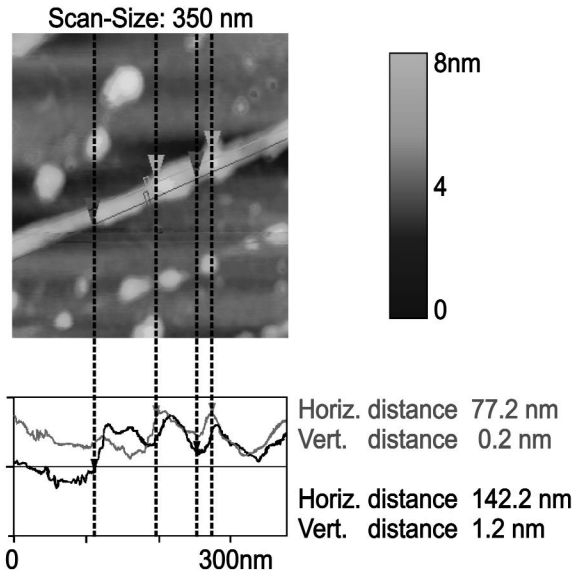

Figure 4. Fibrin strand imaged by AFM in the '"tapping mode" on silicon (4 µg/ml fibrinogen). The two adjacent profiles show the displacement of neighboring fibrin molecules.

cules on mica and fibrin strands on silicon were observable (Figure 5). This exceptional example of an isolated fibrinogen molecule shown in Figure 1 has a length of about 52 nm. An image of the neighboring fibrinogen molecule is evident in the left corner of the outer node. The two profiles of the fibrinogen molecule shown in Figure 2 show the typical trinodular structure of the protein even more clearly. The diameter of the two outer nodes is 18 nm, and the smaller middle node has a diameter of 14 nm. The absolute values taken from the AFM image are significantly greater than those values determined by scanning electron microscopy (SEM) [4]. These observed deviations result from the hydration of the protein when exposed to air, which substantially influences the size of the molecules. In contrast to SEM imaging under vacuum conditions, AFM imaging under air preserves the hydration, lead-ing to significantly higher absolute values. The AFM images also reveal a slight S-shape of the fibrinogen molecule, which is slightly evident in Figure 2. This deviation from a straight trinodular structure is attributed to the point symmetrical arrangement of oligosaccherides at the outer nodes of the fibrinogen molecule [4].

Increasing the fibrinogen concentration to 4 µg/ml with silicon led to a polymerization of fibrin monomers, similar to that of the natural coagulation process. A honeycomb-like structure built by crossing fibrin strands covers areas greater than 1 µm in diameter (Figure 3). Evaluating the orientation of the fibrin molecules where they cross each other (using AFM images) verifies results obtained by transmission electron microscopy [5]. Four fibrin molecules are involved in forming the characteristic node, which is the building block of the observed ring structure. The irreversible conversion from fibrinogen into fibrin is not so much correlated with a change of the overall protein structure imaged by AFM. More important appears to be that the charge of the middle node is changed from -8 to +5, while the negative charge of the outer nodes (-4) is unaffected, in fibrin formation [4]. Thus, the contact activation of fibrinogen causes a fundamental change in the electrostatic interactions between the molecules. The characteristic ring structures are indicative of this fact. The consequences of the electrostatic interaction between the fibrin molecules become more obvious in Figure 4 where the structure of a single fibrin strand is depicted together with two adjacent height profiles. Neighboring molecules are displaced parallel to each other. The electrostatic attraction between the central and outer nodes of neighboring fibrin monomers results in this particular arrange-

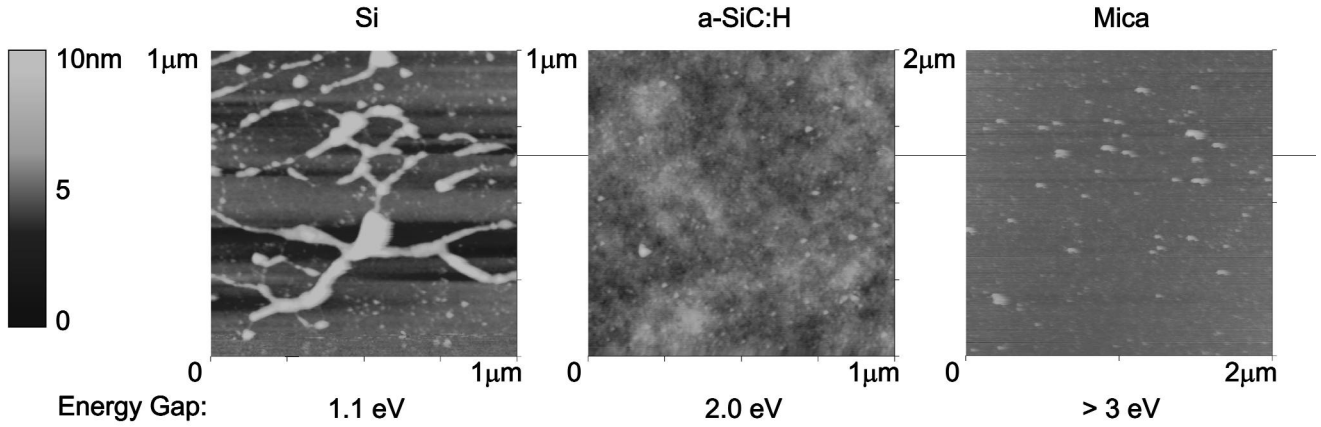

Figure 5. Scanning force microscopy-images done in the "tapping mode" of Si-, a-SiC:H- and mica-surfaces incubated with fibrinogen solution (6 µg/ml). The energy gaps of the materials are 1.1eV (Si), 2.0eV (a-SiC:H) and >3eV (mica).

ment of fibrin strands that are up to 1 µm in length.The correlation between the electronic properties of artificial materials in contact with blood and the contact activation of fibrinogen [1] was analyzed with AFM imaging of three semiconducting materials with different energy gaps. In Figure 5, three typical AFM images of the surfaces incubated with a 6 µg/ml fibrinogen solution are depicted. On silicon, wide fibrin networks

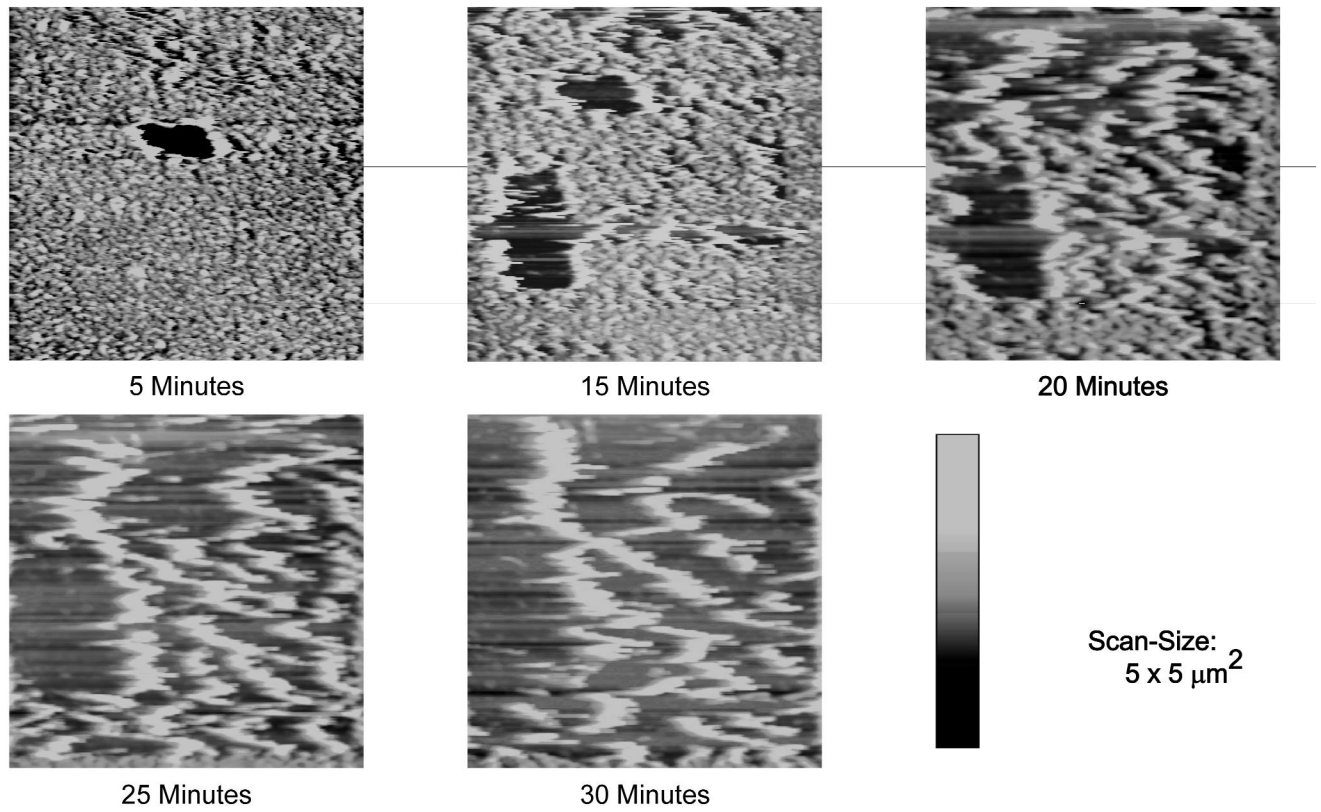

Figure 6. Development of fibrin strands imaged at the silicon-solution phase boundary by AFM in the "contact mode". Physiologic saline solution with 6 µg/ml fibrinogen.

were imaged (Figure 5, left panel) as previously shown in Figure 3. In contrast to the results obtained on silicon surfaces, only single molecules and small clusters of fibrinogen were observable on a-SiC:H (Figure 5, middle panel) and mica (Figure 5, right panel) under the same experimental conditions. The recognizable structure on the a-SiC:H surface in the middle panel of Figure 5 is solely due to the amorphous character of the a-SiC:H substrate prepared in a plasma-enhanced chemical vapor deposition (PECVD) process. The different electronic properties of the three semiconducting materials provide a reasonable explanation for these observations. On silicon, which has the smallest energy gap (E_{gap} = 1.1 eV), the electron transfer is possible and the conversion into fibrin takes place [1]. In contrast, on the substrates mica (E_{gap} > 3 eV) and a-SiC:H (E_{gap} = 2.0 eV) no tunneling into free electronic states is possible, and no conversion of fibrinogen into fibrin occurs. Thus, the AFM images give an indirect indication for the validity of the electronic model of contact activation at the molecular level.

A time-resolved observation of the development of fibrin strands was possible directly at the silicon-solution phase boundary. Stable imaging was possible at low fibrinogen concentrations (0.4 µg/ml only). At higher concentrations the tip and the cantilever were contaminated by adsorbed macromolecules, and the quality of the images obtained was too poor. In Figure 6, AFM images taken over a period of 30 minutes at the same place on a silicon surface are depicted. The homogenous distribution of molecules imaged after 5 minutes changes into vertical strands after 15 minutes. The comparison with the AFM images taken under air (Figures 3 and 4) and the stable and reproducible observation over a period of half an hour are strong indications for the development of surface-bound fibrin strands on silicon. In contrast to the results obtained on silicon, investigations on a-SiC:H and mica gave no evidence for the development of fibrin strands. In the AFM images only a homogenous distribution of molecules could be identified, which confirms the results obtained under air (Figure 5).

Discussion

Images of the interaction between fibrinogen molecules and solid surfaces — the key process during contact activation — were made accessible under air and fluid with AFM methods. Isolated fibrinogen molecules with their characteristic trinodular structure were sketched with molecular-level resolution. In contrast to the dimensions measured with SEM in studies [4], preserved hydration of the proteins during AFM imaging led to the determination of larger absolute values that represent a more realistic measurement of the macromolecule.

Fibrin strands and networks were seen under air with molecular-level resolution at the higher concentrations of fibrinogen used in the study. Fibrin strands were also reproducibly imaged directly at the silicon-fibrinogen phase boundary containing solution. Though the resolution under fluid is not at a molecular level, the observations under air could be verified in the physiological environment. A significant difference in the charge distribution causes electrostatic attraction between fibrin molecules, and electrostatic repulsion between fibrinogen molecules [4]. Thus, the formation of strands and the displacement of neighboring molecules within the strands provide evidence of the conversion of fibrinogen into fibrin. The characteristic ring structures in the fibrin networks observed in many AFM images are another indication of the changes in electrostatic interaction caused by the contact activation of fibrinogen.

The AFM measurements of the three semiconducting materials — silicon, amorphous phosphorous-doped silicon carbide, and mica, with energy gaps of approximately 1.1 eV, 2.0 eV and 3 eV respectively — show the interdependence between electronic properties and contact activation of fibrinogen [1]. Only on silicon, the material with the smallest band gap, was fibrin observed; whereas on the other materials, only single fibrinogen molecules and clusters were resolved. The different electronic properties of the three materials tested led to differences in contact activation as observed on a molecular level by AFM. Thus it was found that it was possible for electrons to tunnel from fibrinogen into the free electronic states of the solid only on silicon. The results obtained by AFM on a molecular level give further evidence for the validity of the electronic model of contact activation of fibrinogen on a molecular level, while explaining the very good hemocompatibility of a-SiC:H-coated implants such as stents.

These findings show that AFM methods are a powerful molecular approach in attaining an improved physical understanding of the contact activation of fibrinogen. Studies are currently in progress for applying

AFM methods, especially the "tapping mode", for molecular-level resolution in electrolytes, and extending AFM studies to electrochemically controlled surfaces. New insights into the correlation between physical properties of materials and the processes involved during contact activation will lead to a better understanding of the roles played by fibrinogen and other blood plasma constituents.

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