Fibrinogen Molecular Images by Scanning Force Microscopy to Study the Mechanism of Contact Activation

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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 induction by biochemical processes, contact activation of fibrinogen is also stimulated by an electron transfer from the fibrinogen molecule to a solid surface in direct contact with the blood. Thus, the structural and electronic properties of fibrinogen and artificial materials both contain thrombogenic potential. Scanning force microscopy (AFM) is extremely useful for studying these processes. This method allows for a structural analysis of biological objects and solid surfaces on a nanometer scale. In the presented study, the structure of fibrinogen was investigated at a molecular scale, showing its typical trinodular structure which is approximately 52 nm in length. At higher concentrations, fibrin networks were imaged on silicon. The analysis of their molecular structure shows that the strands are built of fibrin molecules displaced parallel to each other. The correlation between the potential of contact activation and electronic properties of fibrinogen and material was studied using AFM measurements on semiconducting materials with different energy gaps. On silicon, the material with the smallest energy gap, fibrin was observable, whereas on the materials with higher energy gaps, only single fibrinogen molecules and small clusters of fibrinogen were observable.

Key Words

fibrinogen, fibrin, contact activation, scanning force microscopy

Introduction

A deep 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 structural and electronic properties of the implant surface and the plasma constituents together determine the hemocompatibility of the device [1].

The irreversible conversion of fibrinogen to fibrin is of particular importance for contact activation. This process was studied with the scanning force microscopy (AFM) technique. 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; and the interdependence of the electronic properties of artificial materials and the blood that leads to the development of fibrin strands at the implant surfaces. AFM performed in the "tapping mode" 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.

Methods

Investigations were done with the Multimode-AFM connected to the Nanoscope III controller from Digital Instruments GmbH. To minimize lateral forces and to allow for stable imaging of biological objects, the "tapping mode" was used [5]. Cantilevers were beam-shaped silicon nanoprobes. Imaging was done under

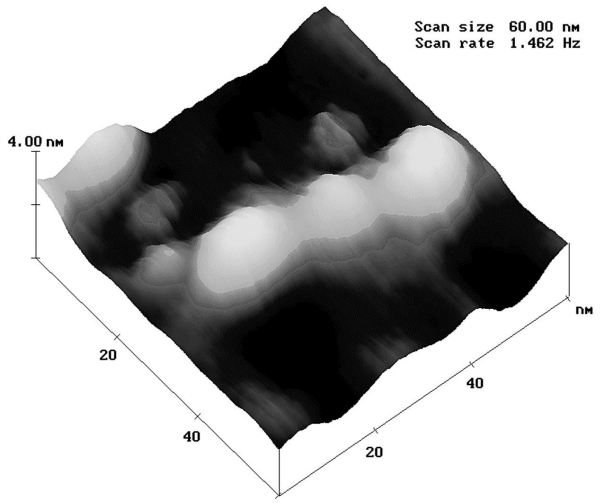

Figure 1. Isolated fibrinogen molecule imaged by AFM in the "tapping mode" on silicon (1 mg/ml).

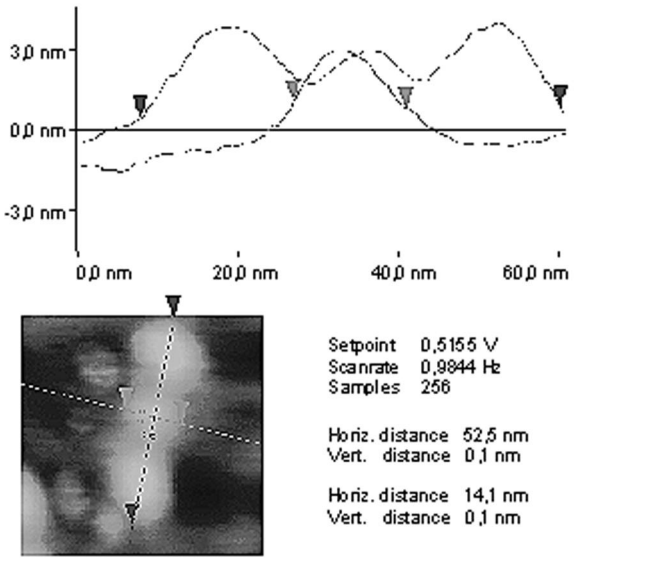

Figure 2. Isolated fibrinogen molecule imaged by AFM in the "tapping mode" on silicon with the corresponding profiles of the protein.

air. Substrates were freshly cleaved mica and silicon wafers in the [111]-orientation. Additionally, phosphorous-doped amorphous silicon carbide (a-SiC:H-) was chosen as a prototype of a hemocompatible material [1]. 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 step by dissolving 1 to 6 mg/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 airdried.

Results

On mica and silicon, reproducible imaging with submolecular resolution of single molecules was possible at low fibrinogen concentrations of 1 mg/ml (Figure 1). This imaging revealed the typical trinodular structure of fibrinogen. At higher concentrations, only small clusters of fibrinogen molecules on mica and fibrin strands on silicon were observable (see 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 resolve 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) [3]. 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, leading 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 [3].

Increasing the fibrinogen concentration to 4 mg/ml on 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 mm in diameter (Figure 3). The evaluation of the orientation of the fibrin molecules at the points where they cross, determined by an analysis of the AFM images, verifies results obtained by transmission electron microscopy [4]. It can be seen that four fibrin molecules are

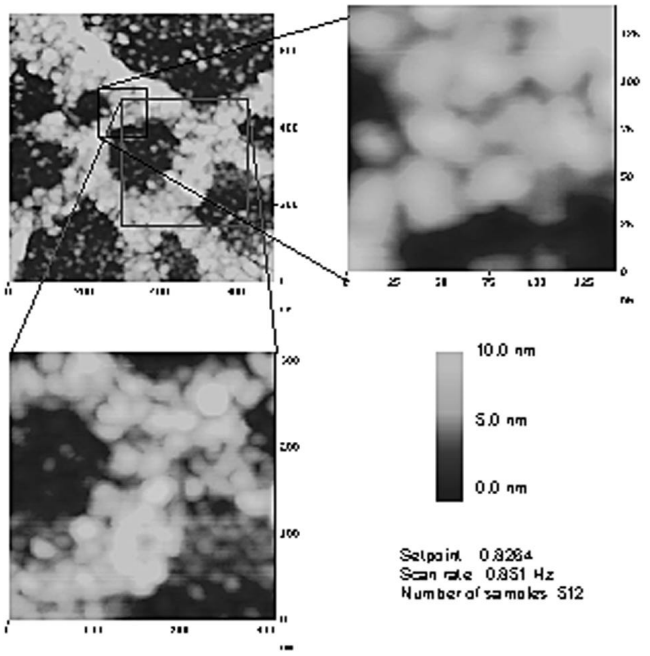

Figure 3. Fibrin network imaged by AFM in the "tapping mode" on silicon (4 mg/ml). In the two sections, single fibrin molecules are clearly resolved.

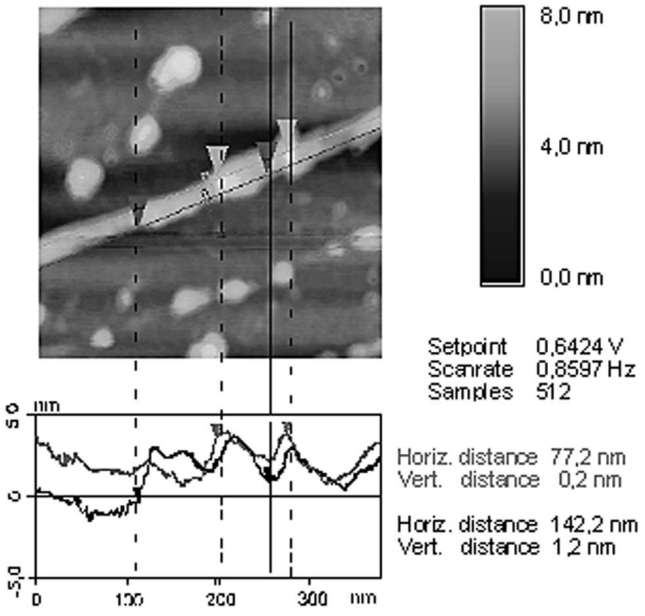

Figure 4. Fibrin strand imaged by AFM in the '"tapping mode" on silicon. The two adjacent profiles show the displacement of neighboring fibrin molecules.

involved in forming the characteristic node that creates the observed ring structure.

The irreversible conversion from fibrinogen into fibrin is not so much correlated with a change of the overall protein structure that can be imaged by AFM. Much more important is the fact that the middle node charge is changed from -8 to +5, while the -4 charge of the outer nodes is unaffected [3]. 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 effect of electrostatic interaction between the fibrin molecules becomes much more obvious in Figure 4, where the structure of a single fibrin strand is analyzed. 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 arrangement of fibrin strands that are up to 1 mm 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. Figure 5 shows three typical AFM images of the surfaces incubated with a 6 mg/ml fibrinogen solution. On silicon, wide fibrin networks were imaged (Figure 5, upper right panel), as shown previously in Figure 3. In contrast, only single molecules and small clusters of fibrinogen were observable on a-SiC:H and mica (Figure 5, lower panels) under

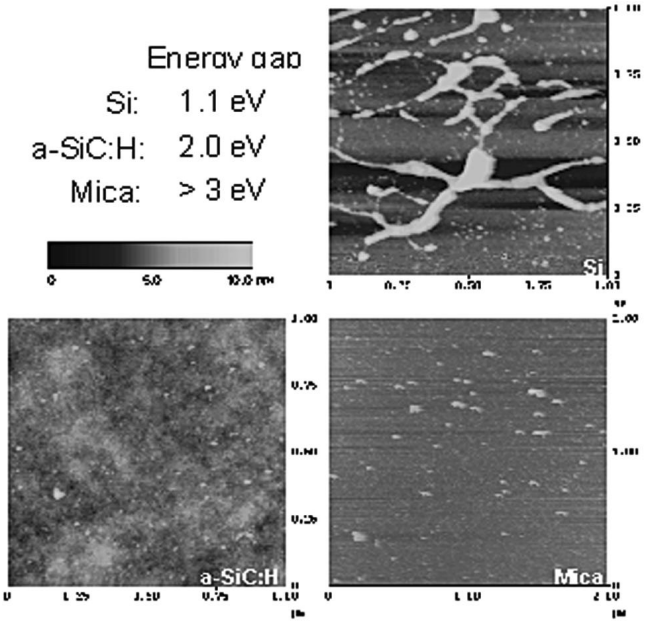

Figure 5. Scanning force microscopy-images done in the "tapping mode" of Si-, a-SiC:H- and mica-surfaces incubated with fibrinogen solution (6 mg/ml).

the same experimental conditions. At this point, it must be noted that the structures on a-SiC:H in the AFM image (Figure 5, lower panel, far left) are artifacts of the plasma-enhanced chemical vapor deposition (PECVD) preparation process of the a-SiC:H-substrates and are not caused by contact activation. The different electronic properties of the three semiconducting materials provide a reasonable explanation for this reaction. Only with silicon, which has the smallest energy gap (Eg) of approximately 1 eV, is the electron transfer responsible for conversion to fibrin at 37°C [1]. In contrast, on mica (Eg > 3 eV) and on phosphorous-doped a-SiC:H (Eg » 2 eV) no tunneling into free electronic states is possible and no conversion of fibrinogen into fibrin occurs.

Discussion

Images of the interaction between fibrinogen molecules and solid surfaces, the key process during contact activation, were made accessible under air with AFM methods. Isolated fibrinogen molecules with their characteristic trinodular structure were viewed with molecular-level resolution. In contrast to the dimensions measured with SEM in studies [3], 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 with molecular level resolution at the higher concentrations of fibrinogen used in the study. A significant difference in the charge distribution causes electrostatic attraction between fibrin molecules, but electrostatic repulsion between fibrinogen molecules [3]. 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 for 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, having energy gaps of approximately 1 eV, 2 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 seen. The different electronic properties of the three materials tested lead 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 solely on silicon.

These results 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 on measurements of macromolecules in electrolytes, and extending AFM studies to metal surfaces that are held at a defined potential.

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. These revelations will lead to new approaches in the development of hemocompatible surface coatings.

References

[1] Bolz A. Physikalische Mechanismen der Festkörper-Protein-Wechselwirkung an der Phasengrenze a-SiC:H-Fibrinogen. Friedrich-Alexander University Erlangen-Nuremberg. 1991.

[2] Lal R, John A. Biological Applications of Atomic Force Microscopy. American Journal of Physiology. 1994; 266: C1-21.

[3] Doolitle R. Fibrinogen and Fibrin. Scientific American. 1981; 246: 126-135.

[4] Mosesson M. Fibrin Polymerization and its Role in Hemostasis. *in*: New Trends in Hemostasis. Harenberg J, Heene D, Stehle G (eds.), Berlin: Springer-Verlag 1990.

[5] Hansma H, Sinsheimer R, Groppe J, et al. Recent Advances in Atomic Force Microscopy of DNA. Scanning. 1993; 15(5): 296-299.