Activation of Cells During Contact with Solid Surfaces

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Summary

The behavior of cells during surface contact is as important as the interaction of solids with free proteins in regard to the medical application. The adsorption of cells to implants can be both desired (growth of a protective layer of vital endothelial cells) as well as undesired (activation of platelets). In order to be able to predict such processes in model calculations, a physical model of the activation of proteins on solid surfaces was generalized to the interaction with cells. In order to verify these hypotheses, the activation of platelets and the growth response of endothelial cells were tested on a metal and a semiconducting substrate, because both processes are of importance to the behavior of implant surfaces during blood contact. Observations made using atomic force microscopy correspond to the proven activation behavior of proteins on solid surfaces of various electronic structures. An activation of cells associated with a disordered adsorption and growth behavior was proven on metals. However, the solid surface adapted to the semiconducting characteristics of the proteins behaves passively, which is in accordance with the physical model of contact activation of proteins at solid surfaces.

Key Words

Biocompatibility, biomaterials, cells

Introduction

The behavior of cells during surface contact is as important as the interaction of solids with free proteins in regard to the medical application. Life-threatening complications such as thrombosis or restenosis after stent implantation, for example, are not only triggered by the interaction between the stent surface and plasma proteins. In fact, cellular components of the blood and cells of the vascular wall very much participate in the underlying mechanisms. Thus, activated platelets release a number of various highly potent substances that are directly involved in blood clotting processes and arteriosclerotic changes [1]. However, adsorption of cells to implants is not always undesired. The growth of a protective layer of vital endothelial cells on a stent is the ideal permanent biocompatible system [2], and the controlled growth of cells on carrier substances is the basis of tissue engineering [3].

An already described physical model of protein activation on solid surfaces through electron transfer processes [4,5] can be generalized to such an interaction with cells. This can be accounted for by the fact that the interaction of cells with their environment is mediated by the membrane proteins of the cellular wall. Lastly, the behavior of individual membrane proteins during surface contact determines the behavior of the larger cell structure, in that domino reactions are triggered in the inner cell. The objective of this investigation was to experimentally verify this hypothesis.

Materials and Methods

Investigations were conducted with two cell types that are especially important for implant surfaces during blood contact. The first type were platelets, which as

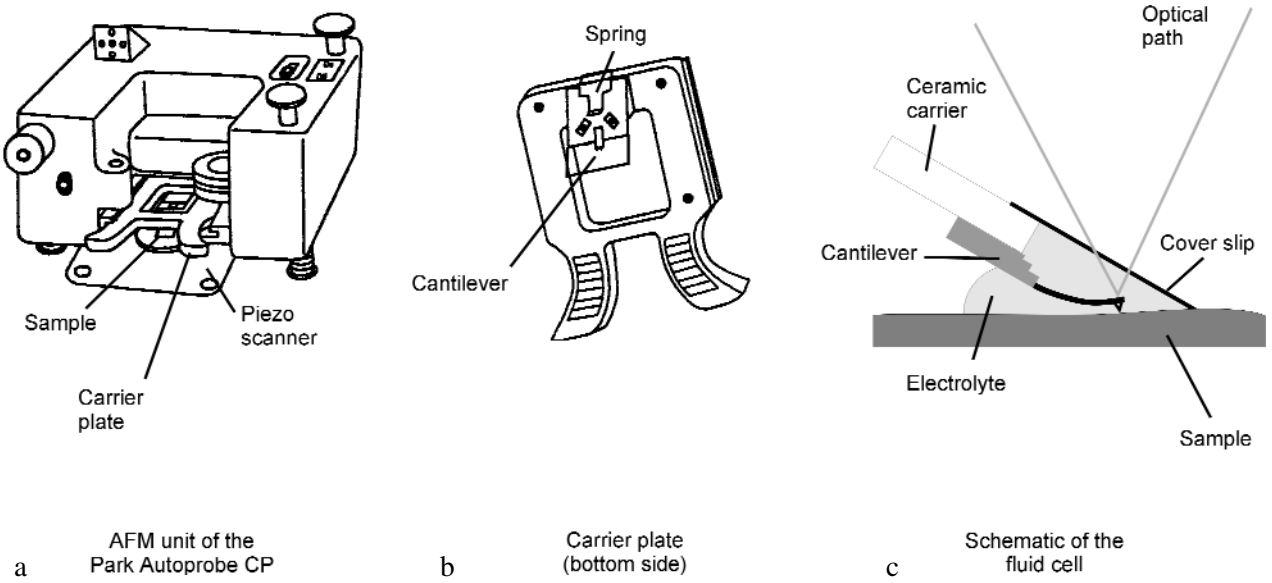

Figure 1. Illustration of the experimental arrangement during tapping atomic force microscopy measurements on platelets and endothelial cells with the AutoProbe-CP system (Park Scientific, USA) in electrolytes.

carriers of numerous highly potent substances directly affect the blood clotting cascade as well as in-stent restenosis. The second type were endothelial cells, which make up the innermost layer of all vessels and are the ideal biocompatible surface for implants in the bloodstream. Compared to proteins, it is even more critical that possible physiological conditions are considered when experimenting with cells. Cells have a metabolism; their behavior is therefore affected even more strongly by environmental conditions. For this reason, atomic force microscopy (AFM) in liquids [6] was used for analysis, which allows one to investigate the cell morphology and cell density directly in physiologic electrolytes.

Atomic Force Microscopy (AFM)

The atomic force microscope consists of a sharp tip, which is attached to a soft cantilever. In contact mode, the tip is simply dragged across the sample and the deflection of the cantilever is monitored. In this case, the tip or surface may be damaged by interaction. This disadvantage is eliminated in tapping mode, where the tip is oscillating in a vertical direction. The measurements were conducted with the AutoProbe-CP system (Park Scientific, USA), which allows images both in contact mode as well as in tapping mode in an electrolyte environment. The central unit of the microscope, made up of the piezo scanner, cantilever carrier plate, and optical system, is depicted in Figure 1. The sample to be tested is attached to the piezo scanner and is scanned opposite the stationary cantilever. The cantilever is fastened to a ceramic plate with superglue, which is clamped to the piezo ceramic element for measurements in tapping mode. The geometric design of the measuring tip is such that the user has an unencumbered view from above of the sample and the cantilever. Correspondingly, an optical microscope with CCD camera is integrated in the system; it sits on a swivel arm to facilitate adjustments of the optical system and positioning of the sample. In order to attenuate mechanical vibrations, the entire microscope is placed on an air-bearing granite slab and put under a glass dome. All measurements were conducted with silicon cantilevers in a triangular format (Ultralever, ThermoMicroscopes, USA). The cantilevers have spring constants between 2.1 N/m and 17 N/m as well as resonance frequencies between 80 kHz and 320 kHz. In order to be able to work with small electrolyte quantities, a micro liquid cell was individually prepared for each measurement. As shown in Figure 1c, the micro liquid cell is produced by the microscope's glass cover (attached to the top of the ceramic carrier), which covers the entire area above the cantilever. The problem of rapid evaporation that occurs at the smallest electrolyte

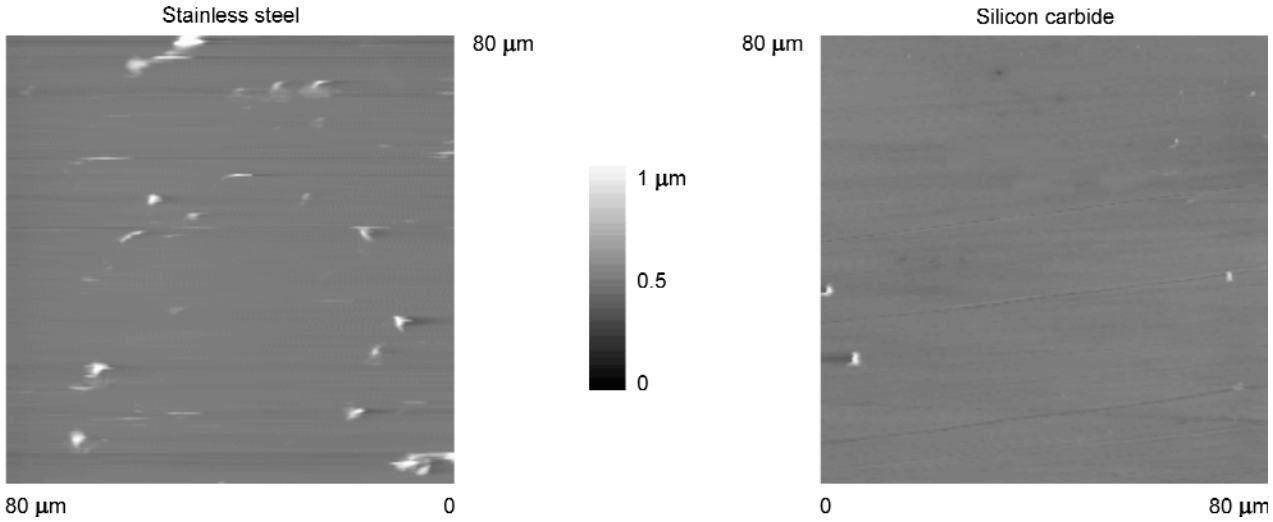

Figure 2. Atomic force microscopy image of 316L stainless steel and silicon carbide (a-SiC:H) after 90 s of contact with a platelet electrolyte (HEPES-Tyrode Buffer; pH 7.3; 10^5 platelets/µl).

volumes is prevented with this design, as are vibrations of the droplet surface due to raster movement, which make stable optical detection of a cantilever deflection impossible.

Activation of Platelets

Platelets play a central role both inside the physiological blood clotting cascade as well as during pathophysiological processes in vessels [1]. With a diameter of 2 – 4 mm, they are the smallest corpuscular blood components and are present in the peripheral blood in a concentration of 150,000 – 300,000 ml^{-1}. Platelets do not have a cell nucleus, i.e., they are practically incapable of synthesizing proteins. They are thus not cells in the classical sense, but rather transport vehicles for various substances. In the inactivated state, they exhibit the typical disk-shaped platelet form. Activation through surface contact or by antagonists such as thrombin results in a characteristically pronounced change in form along with formation of pseudopodia, which constitute protuberances of the plasma membrane. A change in form is triggered when a critical intracellular Ca^{2+} concentration is exceeded. In activated state, the platelet surface is enlarged by approximately 60%, which enables increased interaction with the environment. During the form change, the platelet secretes stored substances, which directly impact a number of various physiological mechanisms. For example, activated platelets release potent, cytokine-like, inflammatory substances, a process that changes the adhesion properties of endothelial cells and thus benefits the migration of monocytes in the vascular wall. In addition, activated platelets stimulate the migration of smooth muscle cells of the vascular wall. Both effects directly influence arteriosclerotic changes of the vascular wall and are suspected to contribute to restenosis after stent implantation. Activation of platelets on implant surfaces should therefore be considered as alarming.

In order to investigate the effect of the electronic properties of solid surfaces on the activation of platelets, metal and semiconducting substrates were tested using atomic force microscopy after contact with a platelet-containing electrolyte. The platelets stem from a concentrate used in clinical practice in the event of insufficient platelets. They are present in a concentration of 10^5 μl^{-1} in the HEPES-Tyrode buffer used as electrolytes (139 mmol NaCl, 2.9 mmol KCl, 1 mmol $MgCl_2 \cdot 6\ H_2O$, 12 mmol $NaHCO_3$, 0.34 mmol NaH_2PO_4, 20 mmol HEPES) at pH 7.3. Surgical steel (316L) was used as a metal sample, and 316L stainless steel coated with amorphous silicone carbide (a-SiC:H) was used as a semiconducting sample. While the activation of proteins is observed on the bare 316L steel [7], the electronic properties of a-SiC:H are optimized as regards a passive behavior towards proteins [8]. Immediately before the AFM measurements, the substrates are placed in contact with the newly attached platelet electrolytes for 90 s and then rinsed with buffer solution.

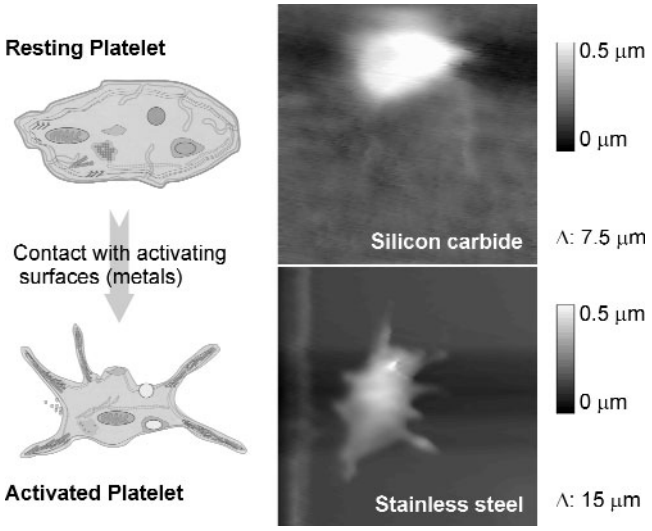

Figure 3. Atomic force microscopy image of silicon carbide (a-SiC:H) and 316L stainless steel after 90 s of contact with a platelet electrolyte (HEPES-Tyrode Buffer; pH 7.3; 10^5 platelets/µl).

Growth Behavior of Endothelial Cells

Endothelial cells line the entire human vascular system and make up the boundary between blood and tissue. They take on central functions during: nutrient intake; migration of cells into tissue for immune defense purposes; and regeneration of arteries and veins [9]. Endothelial cells in the entire vascular system have a potential of cell division and cell movement. In the event of a vascular injury, proliferation and migration of the directly adjoining endothelial cells take place until the endothelium is again completely covered. Endothelial cells are even capable of forming vital endothelial layers on implant surfaces. In clinical practice, this property is used to populate artificial vessels with autologous endothelial cells before their implantation in order to simulate an intact endothelium and thus prevent early vascular occlusion [10]. Because endothelial cells make up the physiologic boundary layer to the blood in the entire vascular system, the formation of a complete endothelial layer on vascular implants is generally viewed as an ideal biocompatible surface [2]. The positive properties of the endothelium are due to the complex structure of the cell membrane of endothelial cells. Various proteins, glycoproteins, glycolipids, and proteoglycans are built into the lipid bilayer. The anionic groups of the proteoglycan are dominant, so that cell surfaces that are in contact with blood have a negative excess charge [11]. Therefore, electrostatic repulsion prevails between the negatively charged blood proteins (at physiologic pH value) and the vascular wall. Due to these properties, one should aim – in contrast to platelets – for an adsorption of vital endothelial cells on an implant surface.

Analogous to the tests with platelets, the relationship between the electronic properties of a solid surface and the growth of endothelial cells were investigated using light and atomic force microscopy. The tests were conducted with endothelial cells from the aorta of a cow. The cells originated from the second passage of a stable cell line (CCL-209, American Type Culture Collection (ATCC), USA). The culture medium used (Modified Eagle Medium) contained 80% MEM (ATCC's Minimum Essential Medium), 20% fetal cow serum, and the additives sodium hydrogen carbonate, sodium pyruvate, and penicillin/streptomycin. The cells were in contact with the culture medium at all times during the test, even during microscopy. Surgical steel (316L) was used as a metal substrate, and 316L stainless steel coated with amorphous silicone carbide (a-SiC:H) was used as a semiconducting substrate. At the start of the test, the endothelial cells were placed on the substrate with a surface of 1 cm^2 and then incubated at 37 °C in an atmosphere enriched with CO_2.

Results

Activation of Platelets

The behavior of platelets upon contact with bare 316L steel and a-SiC:H clearly differs. Numerous platelets can be seen on the metal substrate on the AFM image (Figure 2 left). In contrast, the semiconducting substrate exhibits only a few adhering platelets (Figure 2 right). The diameter of the platelets is approximately 2 – 4 µm and thus concurs with the known value [1]. 316L steel and a-SiC:H not only differ in their number of adsorbed platelets but also in their activation state. In the AFM images of individual platelets on 316L steel, one can clearly see a geometric structure with pronounced pseudopodia, which is characteristic of the activated state. The lower part of Figure 3 contains an example of an AFM image of a platelet on 316L steel together with an illustration of the morphology in activated state. The consistency of the structures is clear. By comparison, with a-SiC:H the adhered platelets remain in a resting state. The disk-shaped platelet form that is characteristic for this state can be clearly seen in the upper part of Figure 3.

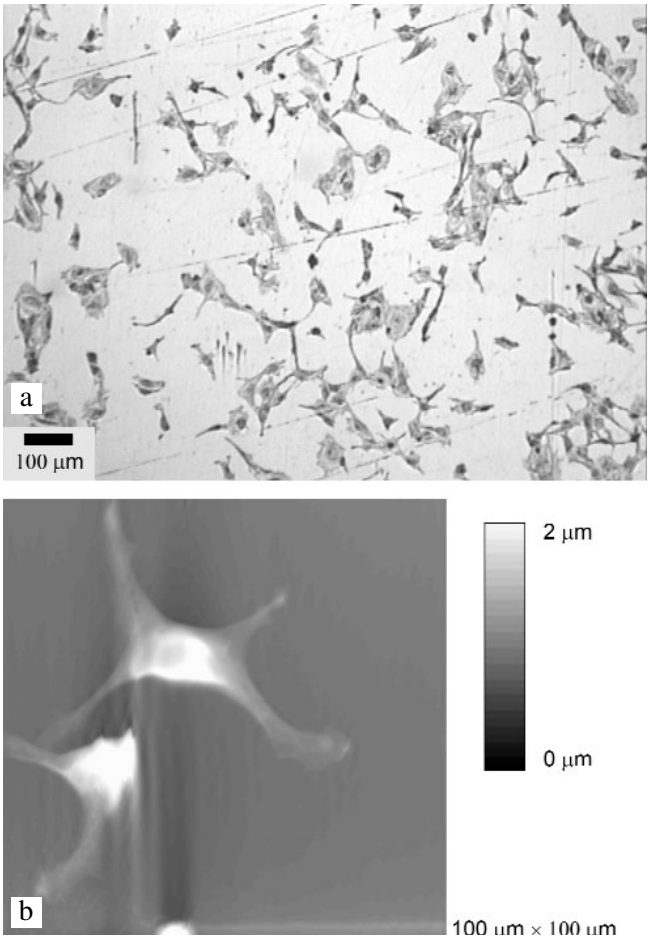

Figure 4. Endothelial cells from the aorta of a cow on 316L stainless steel after a 24-hour incubation period at 37 °C in a culture medium. The images taken with light microscopy (a) and atomic force microscopy (b) show isolated endothelial cells with strongly pronounced pseudopodia.

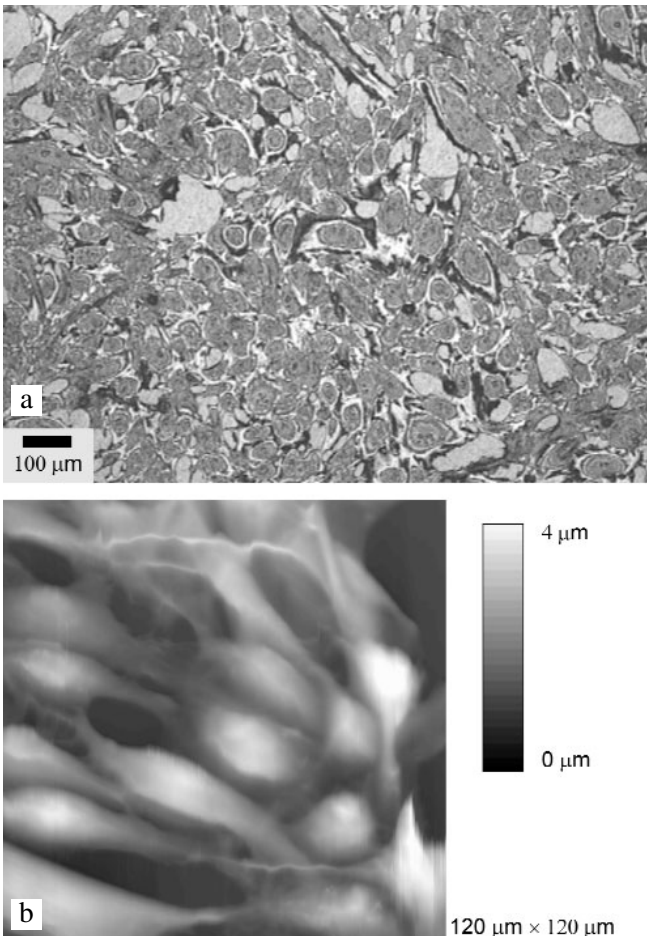

Figure 5. Endothelial cells from the aorta of a cow on 316L amorphous silicon carbide (a-SiC:H) after a 24-hour incubation period at 37 °C in a culture medium. The images taken with light microscopy (a) and atomic force microscopy (b) show a protective layer of endothelial cells with a characteristic pavement structure.

Growth Behavior of Endothelial Cells

Endothelial cells exhibit clear differences in growth behavior on metal and semiconducting solid surfaces. After 24 hours contact of the cells with bare stainless steel only some endothelial cells are present when viewed with light microscopy (Figure 4a). The majority of the cells is isolated on the surface. Cell-to-cell contacts between adjoining cells are rare. The endothelial cells have pronounced pseudopodia of approximately 30 µm in length (Figure 4b). These protuberances of the plasma membrane are characteristic for cells that wander or are searching for contact to adjoining cells. However, when endothelial cells contact an a-SiC:H substrate a completely different behavior is seen. Under identical experimental conditions, a layer of endothelial cells is formed on the a-SiC:H surface within 24 hours that covers the entire substrate (Figure 5a). The cells are arranged in a squamous (pavement) structure within the formed endothelial layer. The spindle-like geometric shape of the cells is clearly visible in the detailed AFM image in Figure 6. Cell-to-cell contacts have formed between the closely adjoining cells, which are characteristic for a vital endothelial layer. The endothelium that grew on the a-SiC:H surface is very stable and was formed over several days without any changes to the cell structure.

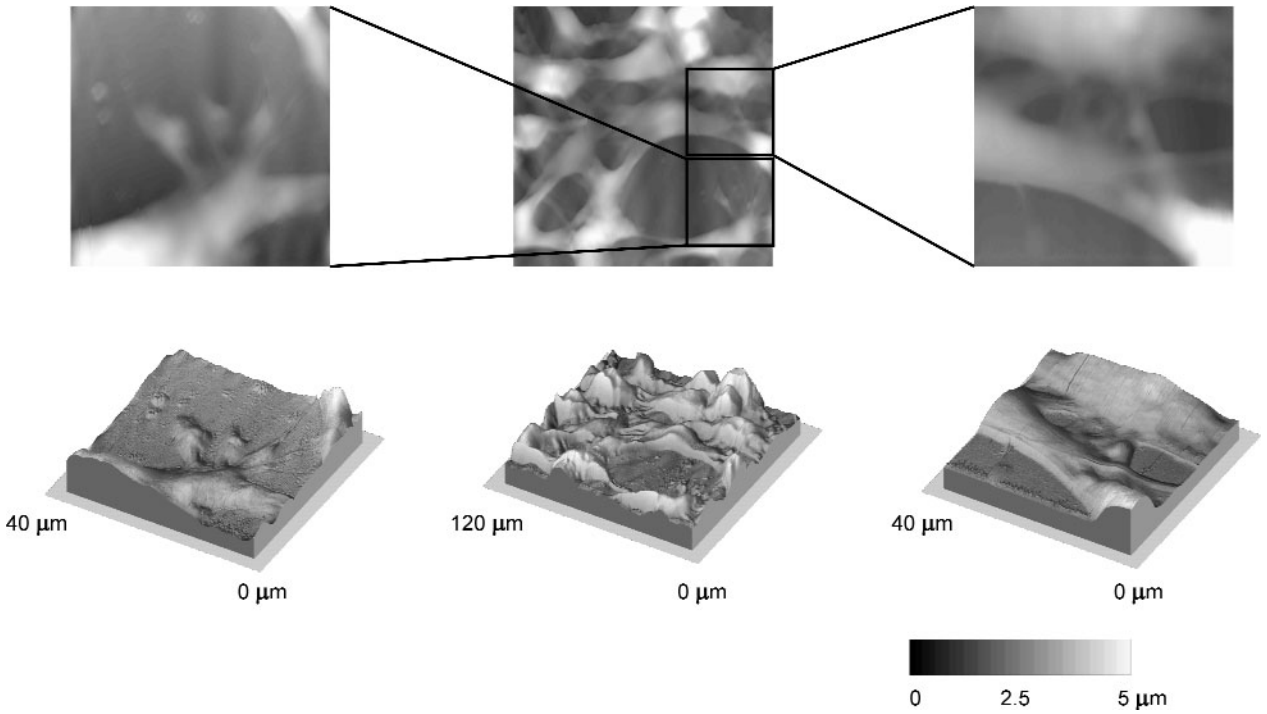

Figure 6. Endothelial cells from the aorta of a cow on 316L amorphous silicon carbide (a-SiC:H) after a 24-hour incubation period at 37 °C in a culture medium. Atomic force microscopy images of the cell-to-cell contacts within the endothelial layer.

Discussion

Based on the existing results it can be generally stated that the behavior of platelets on 316L steel and semiconducting a-SiC:H corresponds to the behavior of proteins. While an activation of the biological structure is seen on metal solid bodies (which is associated with increased adsorption), the solid surface that has been adjusted in its electronic properties behaves passively towards platelets. This conclusion concurs with the physical model of contact activation of proteins at solid surfaces [4] as well as with the hypothesis that the behavior of all the cells is significantly determined by the interaction of the individual membrane protein with the solid surface.

The growth of endothelial cells also exhibits different behavior on 316L stainless steel and on a-SiC:H. No protective endothelial layer is formed on the metal solid surface. The cells are in a stressed state with significantly pronounced pseudopodia. In contrast, the semiconducting solid surface is completely covered by a vital endothelium after the same amount of time. The a-SiC:H surface behaves as an ideal substrate material towards endothelial cells.

Conclusion

Our observations correspond to the proven activation behavior of proteins on solid surfaces of various electronic structures. An activation of cells associated with a disordered adsorption and growth behavior was proven on metals. However, the solid surface adapted to the semiconducting characteristics of the proteins behaves passively, which is in accordance with the physical model of contact activation of proteins at solid surfaces. Electronic exchange currents between individual membrane proteins and the solid surface thus determine the behavior of the cells as a whole.

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