Smart Material Silicon Carbide: Reduced Activation of Cells and Proteins on a-SiC:H-coated Stainless Steel

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Summary

The implantation of stents in coronary and peripheral arteries has proven to be a feasible and successful strategy in the therapy of stenosis. In the last few years the rate of clinical complications has been significantly reduced due to improvements in the mechanical properties of modern stent designs. However, there is a further need for enhancing clinical success, especially by the use of adapted biomaterials. Restenosis and late effects in severely injured vessels are only two examples of current challenges. Further improvements are only possible if there is a deeper understanding of the bio-physical processes governing the activation of proteins and cells at an implant's surface. By controlling the interactions between the alloplastic implant and the biological environment through the use of a suitable stent coating, it is possible to reduce the rate of platelet aggregation, thrombus formation and proliferation of smooth muscle cells. Based on a physical model of the phase boundary between a biomaterial and proteins, and derived from experimental investigations of the behavior of fibrinogen, prothrombin, platelets and endothelial cells at solid surfaces, the requirements for a passive, i.e., "stealth-like" implant surface have been derived. The electronic properties are fundamentally important, and they must be adapted to the physiologic environment. This model of contact activation has been experimentally proven by using semiconducting, amorphous, hydrogen-rich, phosphorous-doped silicon carbide (a-SiC:H) to match the electronic requirements.

Key Words

Biomaterials, contact activation, blood proteins, endothelial cells, platelets

Introduction

Despite improvements in the mechanical properties of modern stent designs, up to 35 % of complication rates (due to long-term restenosis or late effects in severely injured vessels) are still limiting its therapeutic success [1]. The proliferation of smooth muscle cells – the dominant mechanism of restenosis – resulting in neointimal hyperplasia, is suspected to be triggered by interactive processes at the interface between the stent and the biological environment. Today, the majority of all commercially available stents consist of uncoated 316L medical grade stainless steel. However, stainless steel as well as other metals show unfavorable effects when they come in contact with proteins and cells [2,3]. The interactions of proteins or cells with the implant at the phase boundary are the origin of irreversible processes such as thrombosis, leukocyte activation, cell proliferation and complement activation. The activation, cleavage or deformation of proteins triggers further biochemical reactions that may result in a loss of implant functionality. Therefore it is fundamentally important that a further reduction in the complication rates is improved by the biocompatibility of the surface. The development of new biomaterials must be oriented toward those physical properties that distinguish "non-activating" from "activating" materials.

A successful approach to the construction of a stent is the so-called hybrid design concept [4-6] (Figure 1). This concept is based on the combination of two different materials for the bulk and the surface of the stent, respectively. The aim of the hybrid concept is to independently fulfill the mechanical and biocompatibility requirements of the stent. Stent design and mechanical properties of the bulk structure are influenced by rigidity, plastic deformation, and mechanical stability. The surface properties of the stent are determined by important issues such as biocompatibility (hemo- and cell-compatibility), corrosion resistance, and an optimal adaptation to the physiologic environment.

In this hybrid design concept it is possible to use a material such as 316L stainless steel with its superior mechanical properties but relatively poor hemo- and biocompatibility for the bulk of the stent. The surface of the stent is coated with a material that works like a "magic hat" in order to minimize unwanted interactions with the surrounding tissue and blood, i.e., activation processes of adherent proteins and cells. Silicon carbide in an amorphous, hydrogen-rich, phosphorous-doped modification (a-SiC:H) is such a material with superior biocompatibility [7,8]. Its physical properties, especially the electronic properties, are optimized with regard to a minimal activation of adherent proteins. Stents coated with silicon carbide are already in clinical application, and their complication rates are significantly reduced when compared with uncoated stainless steel stents [9-12]. Thus, silicon carbide with its specifically adapted properties can be described as a "smart material".

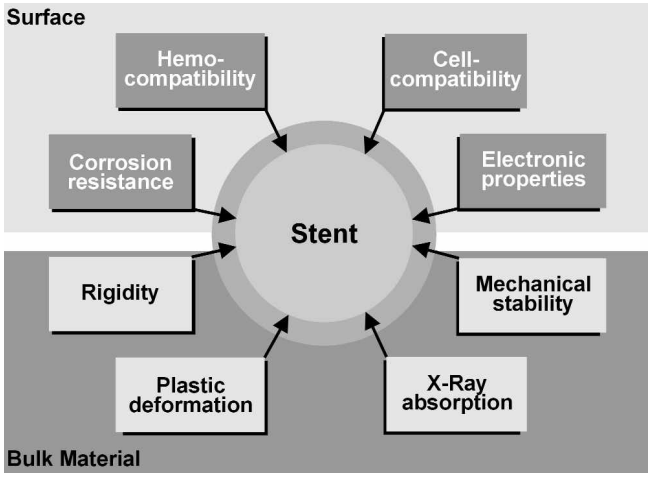

Figure 1. The surface properties of a stent determine the interactions with the surrounding physiologic environment, while properties such as the mechanical performance are determined by the bulk material and the design. The hybrid design of a stent, i.e., a bulk material with a surface coating, allows for optimization with regard to all of the demands.

In the following section, the biophysical model forming the basis for the performance of silicon carbide will be discussed. The starting point will be the physical properties of proteins, which in some ways can be regarded as the fundamental aspects governing the interactions between a biomaterial and the surrounding blood and tissue. Experimental results of two key proteins of the coagulation cascade will be presented to show that the activation of proteins at an implant's surface is triggered by electronic exchange processes. By an adaptation of the electronic properties of the implant's surface to the proteins, it is possible to prevent the activation processes and improve the biocompatibility of bare metals. In the last part of the article, experimental in-vitro results regarding the performance of silicon carbide with respect to contact with cells (namely platelet adhesion/activation on, and endothelialization of uncoated and silicon carbide coated stainless steel), are presented.

Proteins in Contact with Implant Surfaces

An implant's surface is in direct contact with a large number of very different classes of proteins. In the case of a stent the outer surface is in direct contact with proteins that are part of the vessel wall, and the inner surface is in direct contact with proteins that are constituents of the blood. At first glance it seems impossible to design a surface coating which behaves passively towards all proteins. The proteins seem to be too different in nature. But one has to keep in mind that all proteins are built from a set of twenty amino acids, which are the basic building blocks. From a physical point of view a protein can therefore be regarded as a solid that is composed of a set of twenty "atoms", the amino acids. As a consequence, many physical properties of different proteins are quite similar. For the protein activation at surfaces, the electronic properties are of fundamental importance. This important result will be emphasized in the following section for the special case of contact activation of blood proteins.

One of the key processes of the blood-biomaterial interaction is the activation of the coagulation cascade at the phase boundary formed by the implant and the blood. The plasmatic coagulation cascade is a complicated and interrelated series of biochemical processes

(Figure 2). Numerous enzymes catalyze various steps in the cascade, which result in the final conversion of fibrinogen into the fibrin monomer. The adsorption of platelets in the intrinsic system and the injury of vascular cells in the extrinsic systems results in the formation of an enzyme complex, which itself triggers the conversion of prothrombin into thrombin. In the next step thrombin activates the conversion of fibrinogen into fibrin monomers, which polymerize into fibrin strands that stabilize the thrombus.

The observation that fibrin strands are also formed during contact activation of a fibrinogen-containing electrolyte with artificial materials supplies evidence for a direct activation of proteins onto metals [13]. Therefore an additional reaction pathway has to be taken into consideration. Not only the biochemical cascade induced by the adsorption and activation of platelets at the biomaterials surface results in thrombus formation, but also the direct interaction between proteins of the coagulation cascade and the biomaterial at the phase boundary (Figure 2). Two processes that will be discussed are the direct conversions of prothrombin into thrombin, and of fibrinogen into fibrin.

The Activation of Proteins at Implants is Triggered by Electron Transfer Processes

The physical nature of the interactions at the phase boundary between blood and solid surfaces was first studied by Sawyer, Brattain and Body [14,15]. By using a classical electrochemical setup with platinum electrodes, they showed that clotting only takes place at the anode – a direct indication for the participation of electrons at the interaction between blood and metal electrodes. Baurschmidt subsequently proved in his experiments using germanium electrodes, that an electron transfer from the fibrinogen molecule to the germanium electrode caused fibrin formation at the electrode surface [16]. Based on this experimental evidence, a physical model of the activation of proteins encompassing the implant's surface as well as the protein molecules was developed [17].

Because of the participation of electrons, the electronic properties of both the implant's surface and the protein macromolecules have to be taken into consideration in describing the physical mechanisms of contact activation. The first considerations regarding the electronic structure of proteins date back to Szent-Györgyi, who formulated the hypothesis that proteins have semiconducting properties. The semiconducting properties of different proteins were subsequently experimentally verified by different groups [19-22]. For example, Baurschmidt showed that the size of the energy gap was 1.8 eV for the hydrated fibrinogen molecule in solution [16]. Thus, proteins have similar electronic properties to classic semiconductors such as silicon.

The semiconductivity of proteins has the same physical origins as the semiconductivity of amorphous silicon. Due to the large number of structural units in a protein – large proteins such as fibrinogen contain several thousand amino acids (Figure 3a) – it is justified to apply the

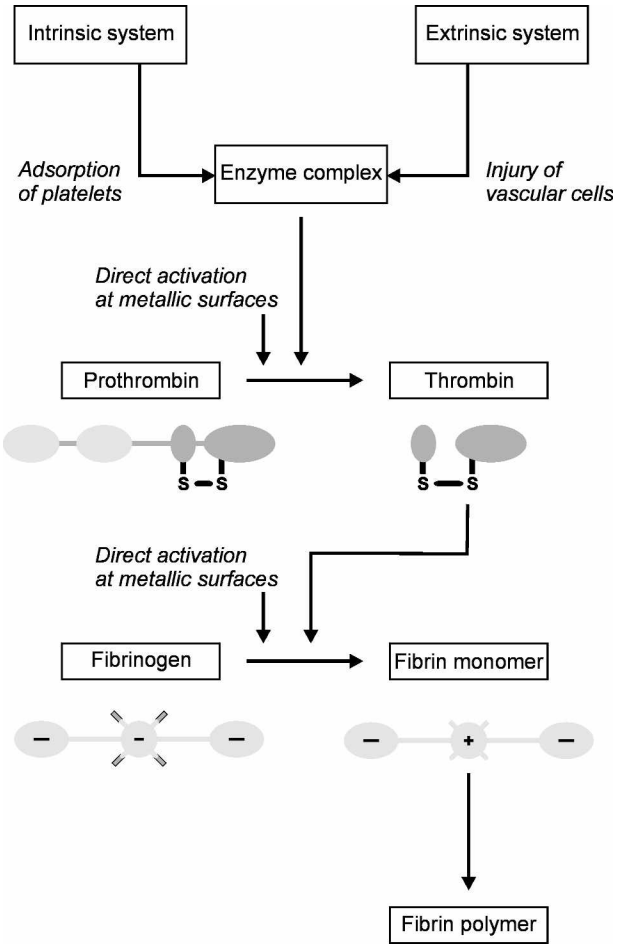

Figure 2. Schematic diagram of the plasmatic coagulation cascade. After stent implantation, thrombus growth via fibrin formation is triggered

- *by the "intrinsic system" due to platelet adsorption,*
- *by the "extrinsic system" caused by even a small injury of the vessel wall,*
- *directly when prothrombin comes in contact with metallic surfaces, or*
- *directly when fibrinogen comes in contact with metallic surfaces.*

physical concepts and description commonly used for solids [22]. Each amino acid shows an electronic structure similar to an atom (Figure 3b). An isolated atom is characterized by discrete electronic energy levels. Starting from the deepest energy level, the electronic states are "filled" with a number of electrons corresponding to the atomic number of the specific element. The electrons with a higher level of energy are farther away from the atomic nucleus than the electrons with a lower level of energy. But the amino acids are not isolated systems; they have common peptide bonds with their neighboring amino acids. As a consequence of the interaction between the outer electrons, the energy levels that are farther away from the atomic nucleus are redistributed across a range of energies, thus forming so-called energy bands (Figure 3c). The detailed distribution of electronic states depends on the nature of the interacting amino acids and the location of all the single amino acids in space, thus being characteristic for the specific protein macromolecule. But protein macromolecules have the electronic structure of a semiconductor in common [19-22]. The energy band containing electrons with the highest energy, also known as the valence band, is completely filled. Between this valence band and the adjacent empty electronic band – the conduction band – there is an energy gap of about 2 eV, for which there are several orders of magnitude less-available electron states (Figure 3c; light grey).

This distribution of electronic states in a protein has important consequences for the contact between proteins and metallic surfaces. In a metal, the energy band containing the electrons with the highest energy is not completely filled. In this case, an electron transfer from the completely filled valence band of a protein macromolecule to the metal is possible because the metal provides a huge number of empty electronic states in the energy range of the valence band of fibrinogen. That is, electrons in the protein are able to occupy empty electronic states with the same energy found in the metal [17,25]. Thus, an electron transfer is possible and an activation of the protein is triggered solely by its contact with the metallic surface.

Experimental Evidence for the Electronic Activation of Proteins

In order to confirm these theoretical considerations and qualitative observations regarding the nature of protein-biomaterial interaction, two key-proteins of the coagulation cascade were studied in detail with respect to the participation of electrons in their activation into solids. These two proteins are prothrombin and fibrinogen. Prothrombin is a blood protein with a molecular weight of about 72,000 Da [26]. The macromolecule is built from five distinct domains: the prothrombin fragments F1, F2 and the amino-acid-chains A, B1, B2. During activation, in the formation of thrombin, a central arginine-isoleucine-bond is broken and the two fragments F1 and F2 are split off (Figure 2). Fibrinogen consists of approximately 3000 amino acids that are arranged in two sets of three non-identical peptide chains [27,28]. During activation, in the formation of fibrin, two pairs of smaller peptides, the fibrinopeptides A and B, are removed from the central node of the protein (Figure 2). Both fibrinopeptides carry a large negative charge resulting in a large difference in the charge distributions of fibrinogen and fibrin. While fibrinogen carries a negative charge on all three nodes, the middle node of fibrin is positively charged, resulting in the formation of strands due to electrostatic attraction.

In order to measure and quantify the electronic exchange current caused by the activation of prothrombin or fibrinogen respectively, cyclic voltammetry at the semiconducting electrode material F-doped SnO_2 (working electrode) was performed. In this electrochemical method, a triangular-shaped potential was applied to a working electrode in contact with an electrolytic solution containing one of the proteins, and the resulting cell current was measured with a potentiostat (Figure 4). To rule out disturbing interactions, the electrodes were located in separate cell compartments connected by Luggin-capillaries. Electron transfer processes were identified as peaks or shoulders in the plot of the current vs. the potential.

The basic idea of this experimental setup is schematically illustrated in Figure 5, which shows the distribution of electronic states at the phase boundary F-doped SnO_2-protein for the cases of thermal equilibrium and anodic polarization of the SnO_2 electrode. Due to an energy gap of 3.8 eV for the SnO_2 electrode, no electron transfer is possible at thermal equilibrium: no empty electronic states are opposite to the filled valence band of the semiconducting protein, (Figure 5, left). An external anodic polarization $V_{Polarazation}$ of the SnO_2 electrode is necessary to shift the electronic states of the electrode and the protein against each other, making an electron transfer possible [17,25] (Figure 5, right).

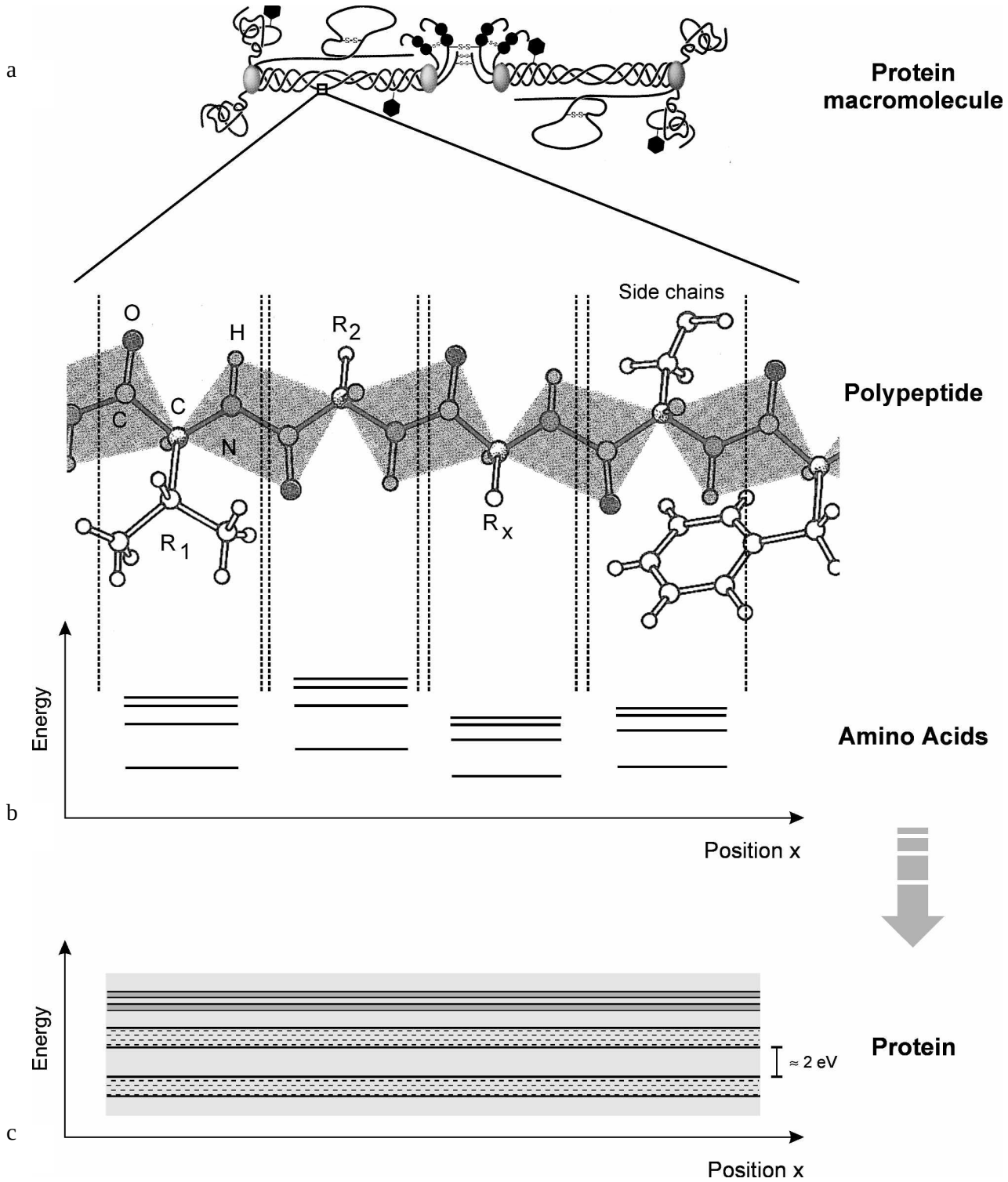

Figure 3. Schematic illustration of the physical origin of the electronic structure of proteins: a) Protein macromolecule with several thousand amino acids. Here is the linear fibrinogen molecule with its three nodular structure. b) The amino acids in the peptide chain have an electronic structure similar to atoms, i.e., electronic states at isolated energies. c) The interaction between neighboring amino acids results in an electronic structure of a semiconducting amorphous solid. The distribution of electronic states is characterized by regions of high and low density. The energy gap between unoccupied and occupied states is about 2 eV for many proteins.

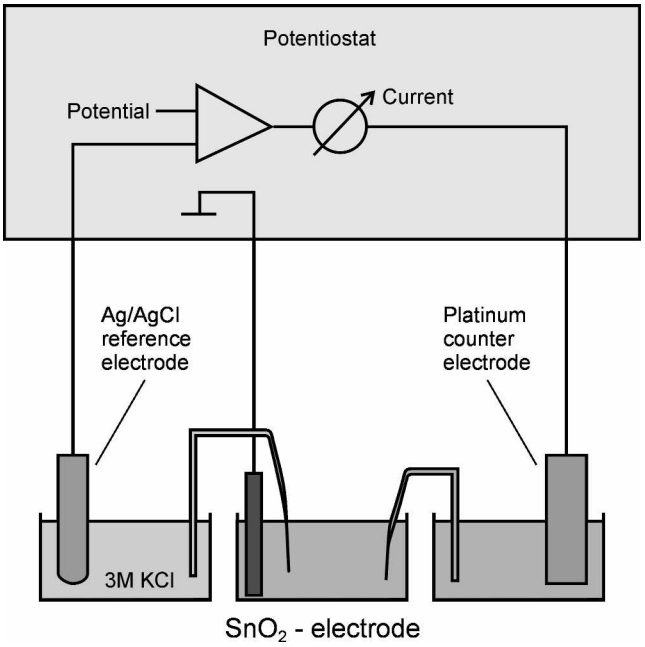

Figure 4. Electrochemical setup used for measurements of the exchange current between protein molecules and an electrode. The three compartments of the electrochemical cell are connected by salt bridges filled with 3 M KCl or protein electrolyte, respectively.

In order to correlate the results of the electrochemical investigations with the activation of prothrombin and fibrinogen respectively, electrolytes used in the electrochemical experiments were analyzed by means of chromatography. The activation of prothrombin was analyzed by gradient High-Performance Affinity Chromatography (HPAC), which allows a direct and specific determination of blood proteins. For the stationary phase, a TSK-Gel-Heparin-PW-column (Tosoh, Japan) with a length of 75 mm and a diameter of 7.5 mm was used in the chromatographic setup. The interaction of surface-bound heparin as the stationary phase with proteins of the coagulation cascade is very specific with regard to the protein type. Due to a linear increase of the NaCl-concentration of the eluent with the analysis time, the interaction between adsorbed proteins and heparin is weakened and a quantitative analysis of protein content is possible. The duration of the concentration gradient is 30 min. The basic eluent contains 20 mM TRIS; the pH is adjusted to a value of 7.5 by addition of HCl. High-Performance Affinity Chromatography was performed at a column temperature of 20 °C. The activation of fibrinogen was analyzed by isocratic Reversed Phase High-Performance Liquid Chromatography (RP-HPLC), which determined the short peptides – the fibrinopeptides. For the stationary phase an RP18-column (Supelco, Germany) with a length of 250 mm and a diameter of 4.6 mm was used in the chromatographic setup. The non-polar regions of the peptides to be analyzed adsorb onto the column due to hydrophobic interaction. In the course of the analysis time the adsorbed peptides can be displaced by non-polar molecules of the eluent, and a quantitative analysis of peptide content is possible. The eluent used for the measurements contains 70 % H_2O,

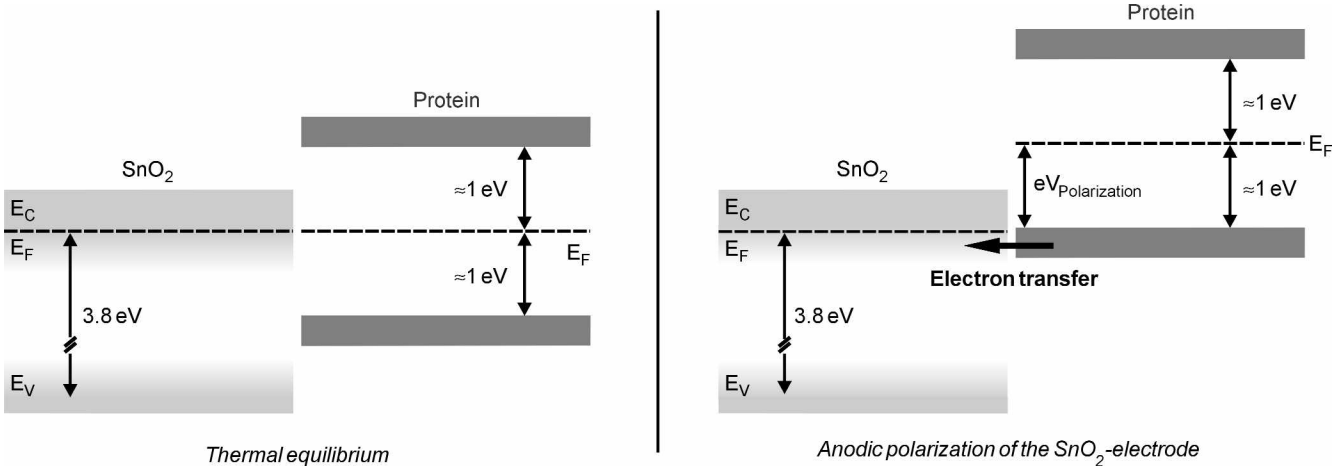

Figure 5. The distribution of the density of electronic states at the SnO_2-protein phase boundary: Thermal equilibrium on the left, and external anodic polarization $V_{Polarization}$ of the SnO_2-electrode on the right. F-doped SnO_2 is a semiconductor with a band gap of about 3.8 eV. Proteins also have semiconducting electronic properties with a band gap of about 2 eV. E_V, E_C and E_F designate the edges of the valence- and conduction-band and the Fermi level.

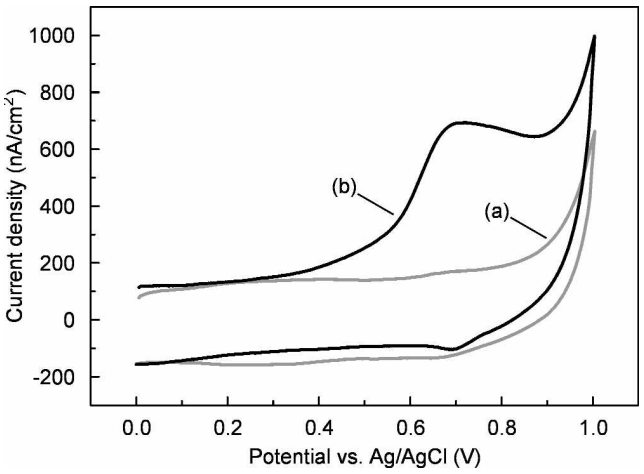

Figure 6. Cyclic voltammograms obtained before (a) and after (b) addition of prothrombin to the electrolyte; 2.5 mg/ml human prothrombin (Sigma, Germany), potential rise of 10 mV/s, 20 mM TRIS-HCl-electrolyte (pH 7.5), 50 mM $CaCl_2$, 154 mM NaCl.

20 % acetonitrile, 9.95 % methanol and 0.05 % TFA (pH 2.2). High-Performance Liquid Chromatography was performed at a column temperature of 20 °C.

The Activation of Prothrombin

Figure 6 shows two voltammograms of an electrolyte with and without prothrombin obtained at an F-doped SnO_2 electrode. As an electrolyte, human prothrombin (Sigma, Germany) was dissolved at a concentration of 10 NIH/ml (National Institutes of Health Thrombin Reference Standard) in TRIS-HCl-buffered saline solution containing 50 mM $CaCl_2$ (pH 8.0). The additional exchange current caused by the presence of prothrombin in the electrolyte is clearly recognizable as a shoulder in the curve. Other electrochemical processes do not superimpose the current, due to the presence of prothrombin. Both the hysteresis caused by capacitive currents that charge the phase boundary, as well as the current above 0.8 V (due to the development of oxygen), show smaller currents in the potential range of interest. The increase in the current-potential curve at a potential of about 0.6 V vs. Ag/AgCl reference is significant in comparison with the protein-free electrolyte. The positive sign of the current shows that electrons are transferred from the prothrombin to the SnO_2 electrode, i.e., it is oxidized at the anodically polarized SnO_2. Furthermore, the asymmetrical shape of the voltammogram is a clear indication of the correlation of the observed electron current with an irreversible electrochemical process.

The left side of Figure 7 shows the corresponding HPAC spectrum of an electrolyte that has been under a cyclic electrical load (0.5 – 0.9 V vs. Ag/AgCl; 10 mV/s) for 17.5 hours. The prothrombin present in the electrolyte at the beginning of the experiment is clearly resolved as a sharp peak at a retention time of about 6.5 min. After 17.5 hours of cyclic electric load,

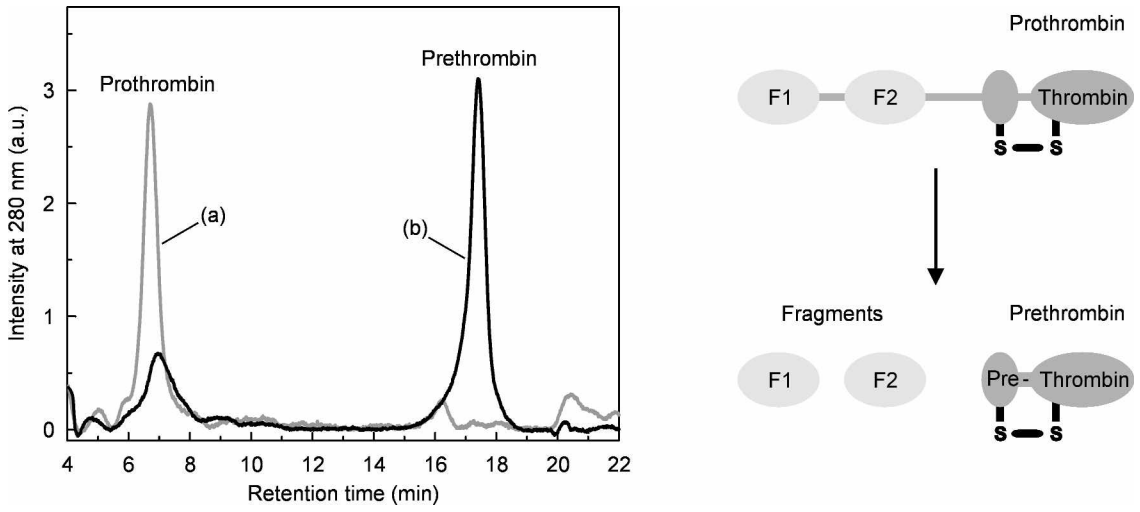

Figure 7. Left) HPAC spectrum of prothrombin containing electrolyte (TRIS-HCl-buffer (pH 7.5), 5 mg/ml human prothrombin): (a) Electrolyte before external potential load. (b) SnO_2 has been in contact with the electrolyte for 17.5 hours under an external potential load (0.5 V – 0.9 V vs. Ag/AgCl; 10 mV/s). Right) schematic illustration of the activation of prothrombin.

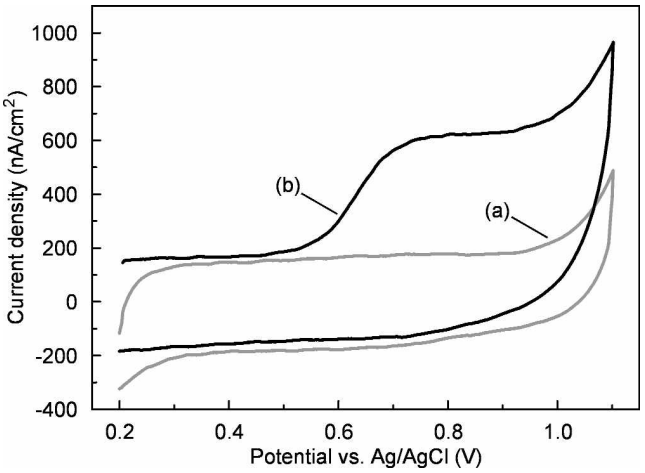

Figure 8. Cyclic voltammograms obtained before (a) and after (b) addition of fibrinogen to the electrolyte; 2.5 mg/ml human fibrinogen (Sigma, Germany), potential rise 10 mV/s, 20 mM TRIS-HCl-electrolyte (pH 7.4), 50 mM $CaCl_2$, 154 mM NaCl.

the magnitude of this peak was clearly diminished and an additional peak was detectable at a retention time of about 17.5 min. This peak is due to the presence of prethrombin in the electrolyte, which is identical to the thrombin except for the difference that the central arginine-isoleucine-bond is not broken [26] (Figure 7, right). A quantitative analysis shows that 47 % of the prothrombin present in the electrolyte is activated, i.e., transformed into prethrombin.

The Activation of Fibrinogen

Figure 8 shows two voltammograms of an electrolyte with and without fibrinogen, obtained with a semiconducting SnO_2 electrode. As an electrolyte, human fibrinogen (Sigma, Germany; type IV from human plasma, 60 % protein, 95 % clottable) was dissolved at a concentration of 2.5 mg/ml in TRIS-HCl-buffered saline solution containing 50 mM $CaCl_2$ (pH 7.4). As in the voltammograms obtained with prothrombin, an additional exchange current setting at a potential of about 0.6 V vs. Ag/AgCl reference, was caused by the presence of the protein. Again, electrons are transferred from the protein to the electrode in an irreversible electrochemical process.

Figure 9 shows a corresponding HPLC spectrum of an electrolyte that has been under a cyclic electrical load (0.4 – 1.0 V vs. Ag/AgCl; 10 mV/s) for 15 hours. Peaks correlated with the fibrinopeptide A (FPA), the fibrinopeptide B (FPB) and the desarginine form of fibrinopeptide B (desArgB), as well as variants of these peptides that differ only in geometry, are clearly resolved. The appearance of the desarginine form of fibrinopeptide B is a well-known effect in in-vitro studies with fibrinogen [29]. A quantitative analysis shows that 80 % of the fibrinogen present in the electrolyte is activated, i.e,. transformed into fibrin by the release of the fibrinopeptides located in the central node of fibrinogen (Figure 9, right).

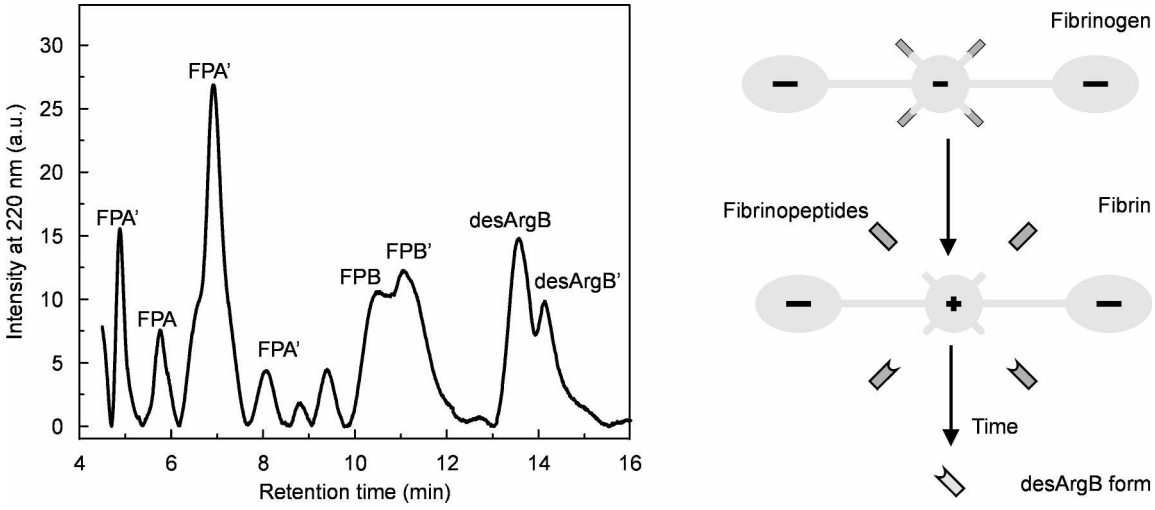

Figure 9. Left) HPLC spectrum of fibrinogen containing electrolyte (TRIS-HCl-buffer (pH 7.4), 5 mg/ml human fibrinogen). SnO_2 has been in contact with the electrolyte for 15 hours under an external potential load (0.4 V - 1.0 V vs. Ag/AgCl; 10 mV/s). Right) Schematic illustration of the activation of fibrinogen. The fibrinopeptide FPB degrades with time to its desArgB form.

From these experimental findings it can be concluded that electron transfer processes over the phase-boundary from proteins to implant surfaces are responsible for the activation of proteins. In the case of proteins of the coagulation cascade, this activation results in thrombus formation. As a consequence, while looking at the plasmatic coagulation cascade (Figure 2), fibrin formation is not only triggered by the extrinsic and intrinsic system, but also directly via the activation of proteins by the surface of an implant. The same mechanism applies to other processes also triggered by proteins.

The Activation of Cells at Implants is Determined by the Membrane Proteins

Risks such as thrombosis and restenosis of stents aren't solely influenced by the interaction of floating proteins with the stent surface. It is also necessary to take into consideration the interaction with cellular constituents of blood and with cells of the vessel wall. Two well-known examples are the activation of platelets due to surface contact and the endothelialization of implants. In order to achieve optimal characteristics regarding reduced complication rates, a passive "non-activating" biomaterial must show less platelet activation and far better endothelialization than metals. But what are the relevant physical properties of a material with an improved performance as it relates to cells? Such a material must have the same physical properties as a material behaving passively regarding floating protein macromolecules; namely, electronic properties that are adapted to the physiologic environment. By looking at the structure and function of a cell membrane this hypothesis becomes apparent (Figure 10). Proteins integrated in the cell membrane, which is formed by phospholipids, regulate cell function. The phospholipids only determine the surface of a cell; they are not relevant to the bio-physical processes. Therefore, proteins in the outer wall of the cell coming into direct contact with the implant's surface, are crucial regarding the interaction with biomaterials. From a physical point of view these membrane proteins can be treated by the same concepts as the other proteins. Thus, they also have the electronic properties of a semiconductor, and electron transfer processes between membrane proteins and implant's surfaces are able to activate a membrane protein, and subsequently the whole cell as a unit.

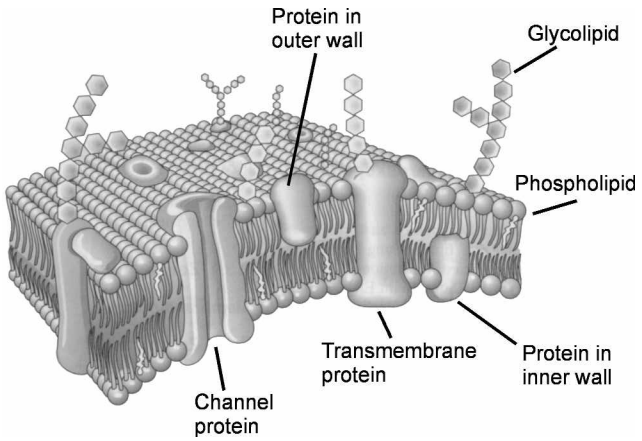

Figure 10. Schematic illustration of the structure of a cell membrane. The interaction of cells with their environment is determined by the proteins located in the cell membrane.

Silicon Carbide (a-SiC:H) – A Non-Activating Surface Coating

Based on these theoretical considerations and experimental evidence regarding the physical mechanisms of protein-biomaterial interaction an optimal biomaterial and deposition process for the coating of a stent's 316L stainless steel bulk material was developed in the last few years [5]. The material is silicon carbide in an amorphous, heavily phosphorous-doped, hydrogen-rich modification (a-SiC:H). The electronic structure of a-SiC:H is adapted to the electronic structure of proteins, thus being the ideal case of a passive, "non-activating" biomaterial.

The deposition of a thin film (80 nm) of a-SiC:H on the bulk structure of a metallic stent is performed by means of a plasma-enhanced chemical vapor deposition technique (PECVD): the gaseous agents silane (SiH_4), methane (CH_4) and phosphine (PH_3) are cracked in a plasma-forming chemically activated species [5]. These activated molecules are able to react at the stent's surface, forming an a-SiC:H-coating that completely covers the bulk structure. In order to achieve a homogenous thickness in the deposited film, the stents are rotated around their longitudinal axis during the PECVD deposition process.

The improved performance of the silicon carbide coating when compared to uncoated stainless steel was already shown in in-vitro studies [7,8]. Proteins and cells show significantly less adhesion and activation on a-SiC:H than on metals. In several clinical studies silicon carbide-coated stents have already shown an

improved performance when compared to bare metals stents [9-12]. In order to supplement these results, additional investigations regarding the interaction with platelets and endothelial cells were conducted.

Reduced Activation of Platelets on Silicon Carbide Coated Stainless Steel

The adhesion, aggregation and activation of platelets at a stent's surface may not only result in an acute thrombosis, but also promote the process of restenosis. In order to investigate the effect of silicon carbide, human platelets in contact with coated and uncoated 316L stainless steel samples were studied in their physiologic environment using scanning force microscopy (AFM) in electrolytes. The imaging of the samples after contact with a platelet-containing electrolyte makes it possible to determine the density of adherent platelets. Furthermore, it is possible to determine the state of a platelet from its geometrical shape. While a resting platelet has a globular shape of about 4 µm diameter (Figure 11, upper left), activated platelets undergo a characteristic change in shape resulting in a hedgehog-like structure with an increased diameter [30] (Figure 11, lower left).

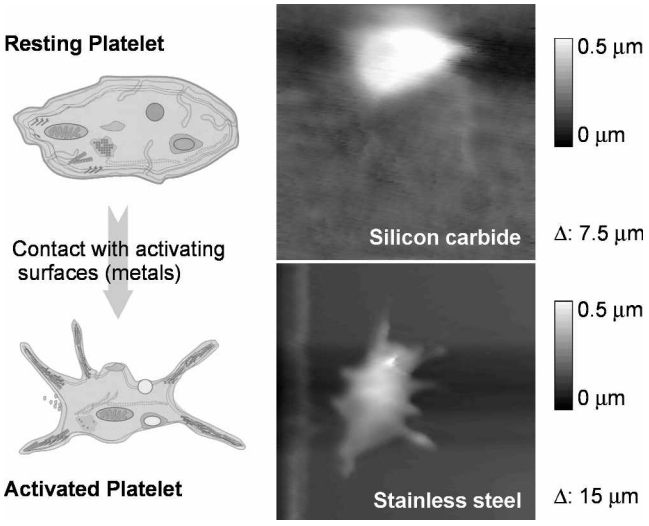

Figure 11. A resting platelet has a globular geometrical shape. The activation of the platelet (for example due to contact with a metal surface), results in a characteristic change in shape to a hedgehog-like structure (left). The two AFM images on the right illustrate this dramatic shape change. On the passive silicon carbide, the platelet remains in a resting state even after contact with the surface. In contrast to this, platelets are activated when in contact with a stainless steel surface.

Experiments were performed with flat a-SiC:H-coated and uncoated 316L stainless steel samples with an area of 1 cm^2. Human platelets were taken from platelet concentrates commonly used in clinical practice. The platelet electrolyte used in the AFM investigations contained platelets in a concentration of 10^5 platelets/µl diluted in a HEPES-Tyrode buffer (pH 7.3). Samples were brought into contact with the platelet electrolyte for 90 s and washed with phosphate buffered saline solution (PBS). Scanning force microscopy imaging was performed in PBS using an Autoprobe CP (Park Scientific, Sunnyvale, CA, USA). On bare 316L stainless steel, a multitude of platelets adhering to the surface is observed. (Figure 12, middle). The majority of platelets have a hedgehog-like shape with marked pseudopodia. (Figure 12, left and right). This characteristic shape for activated platelets is clearly resolved in the AFM image shown in the lower right of Figure 11. Silicon carbide coated 316L stainless steel has a fundamentally different effect on platelets. In the AFM images only a few adherent platelets are present on the surface, and no activated platelets are observed when imaging the surface with different scan sizes (Figure 13). Platelets stay in a resting state even after contact with silicon carbide coated 316L, like the platelet shown in the AFM image (Figure 11, upper left). It can be concluded from these results that the adhesion and activation of human platelets is significantly reduced at silicon carbide coated surfaces.

Improved Endothelialization of Silicon Carbide Coated Stainless Steel

The optimal behavior of a stent material regarding its interaction with the environment is achieved if complete endothelialization with a long-term stable and fully functional layer of endothelial cells is guaranteed within several days. Sound endothelial cells, being the natural interface between blood and the vessel wall, are in a way the perfect coating for a stent [31]. On the one hand, they behave passively with regard to proteins of the coagulation cascade and platelets. On the other hand, they are key players in the mechanisms controlling the growth of smooth muscle cells, i.e., restenosis. In order to investigate the effect of silicon carbide on endothelialization, endothelial cells in contact with coated and uncoated 316L stainless steel samples were studied using AFM.

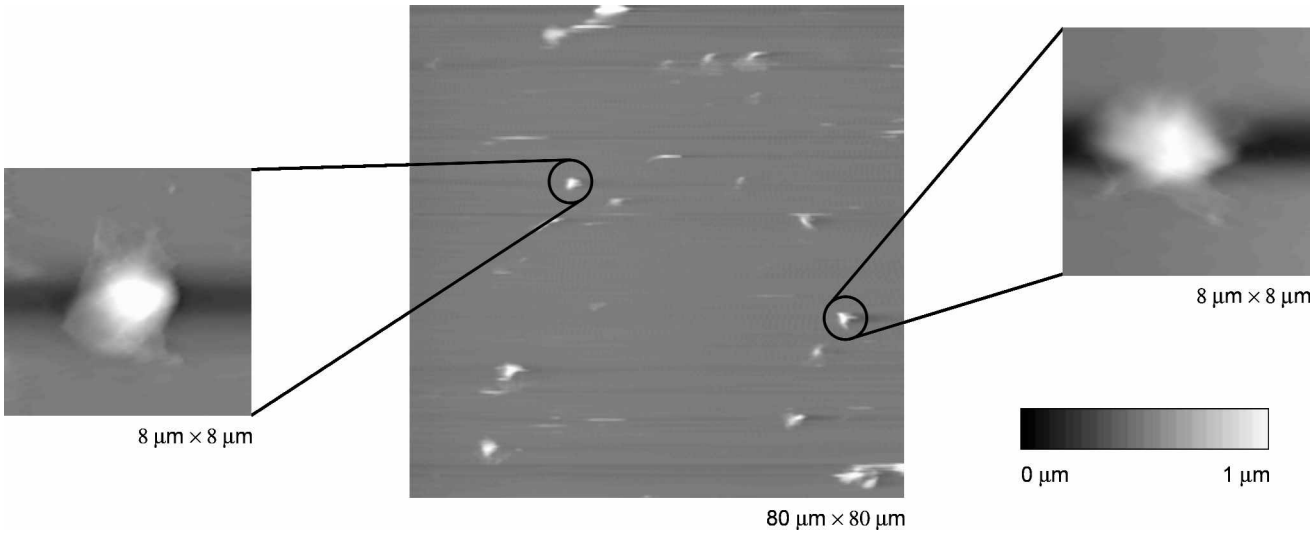

Figure 12. AFM images of 316L stainless steel after 90 s contact with platelet electrolyte (105 platelets/µl, HEPES-Tyrode buffer (pH 7.3). Several activated platelets are adhering to the surface.

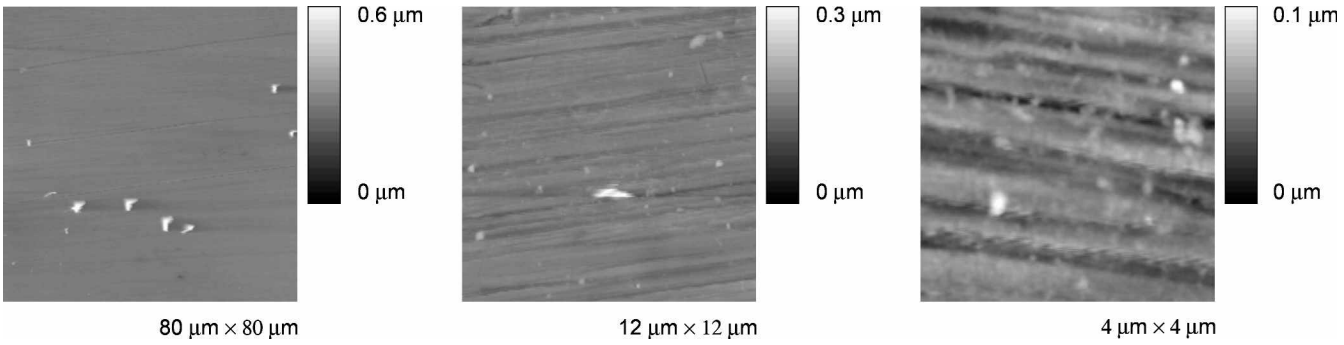

Figure 13. Set of several AFM images with different scan sizes of silicon carbide (a-SiC:H) coated stainless steel after 90 s contact with platelet electrolyte (10^5 platelets/µl, HEPES-Tyrode buffer (pH 7.3). Only a few adherent platelets are present at the surface; no activated platelets are observed.

Experiments were performed with flat a-SiC:H-coated and uncoated 316L stainless steel samples with an area of 1 cm^2. Cardiac bovine endothelial cells (CPAE; ATCC CCL-209) were cultured in MEM medium (80 % Eagle MEM, 20 % FBS), and used after the second passage. Endothelial cells were in contact with the samples for 24 hours. Scanning force microscopy imaging was performed in a MEM Eagle medium at 37 °C using an Autoprobe CP.

After 24 hours of contact between the CPAE and 316L stainless steel, no continuous endothelial layer had formed (Figure 14, upper). The majority of cells had no contact with their direct neighbor. The single endothelial cells showed marked pseudopodia with lengths of about 1 µm (Figure 14, lower). In contrast to these observations, the situation is completely different on the a-SiC:H coated samples. During the same time and under the same experimental conditions, a continuous endothelial layer covering the whole sample had developed (Figure 15, upper). The single endothelial cells showed a sound spindle-like shape. (Figure 15, lower) The endothelium itself showed a characteristic pavement structure. Cell-to-cell contacts had developed between neighboring cells. Thus, the silicon carbide coating also shows a superior performance regarding endothelialization.

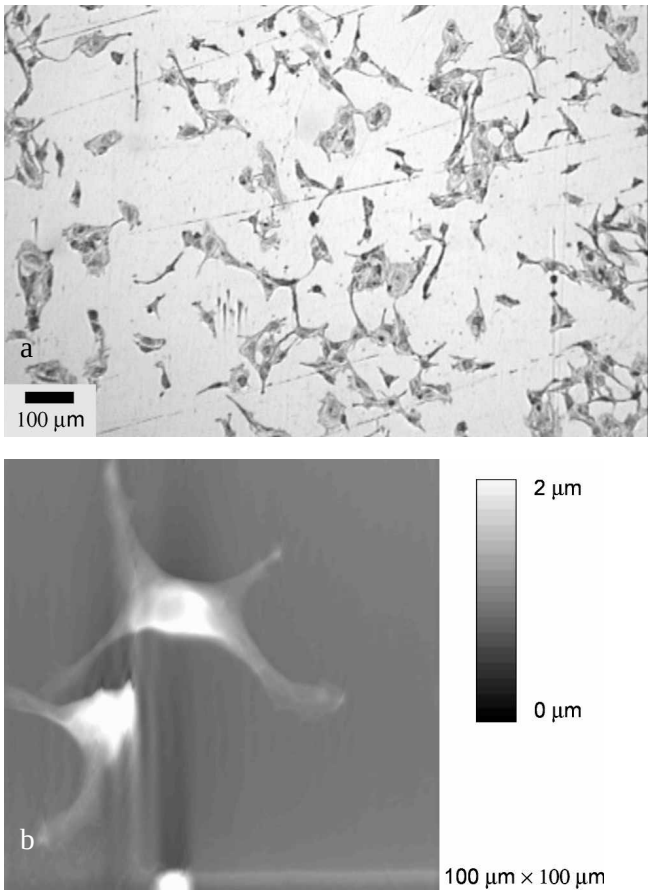

Figure 14. Endothelial cells (CPAE) after 24 hours of contact with 316L stainless steel in modified Eagle medium: (a) Light microscopy image showing only isolated cells on the surface. (b) AFM image showing endothelial cells with marked pseudopodia.

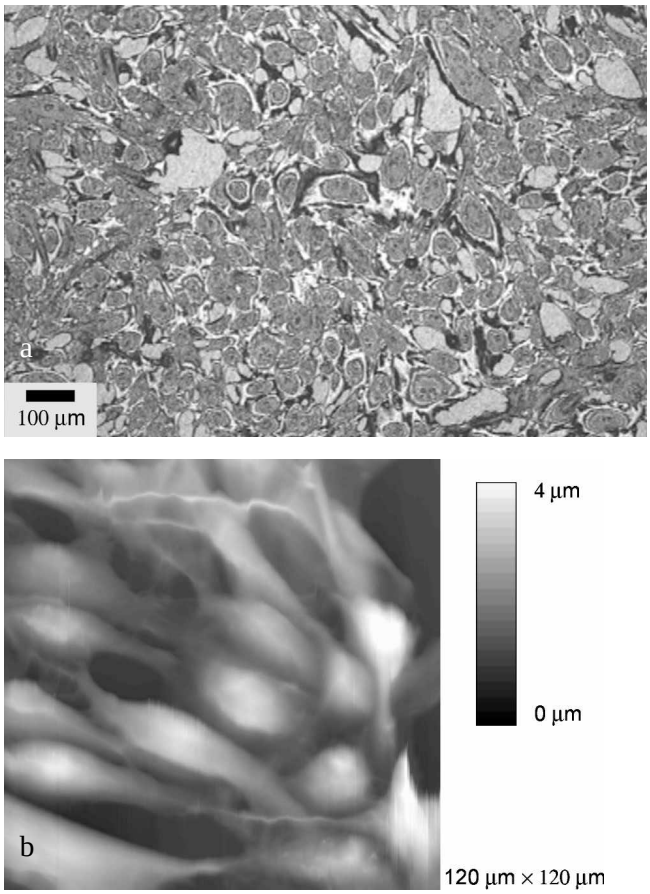

Figure 15. Endothelial cells (CPAE) after 24 hours of contact with silicon carbide (a-SiC:H) coated stainless steel in modified Eagle medium: (a) Light microscopy image showing an endothelial layer covering the silicon carbide completely. (b) AFM image showing endothelial cells with characteristic pavement structure.

Conclusion

The direct activation of proteins and cells on implants is an important reaction pathway that has to be considered in the development of a passive, "non-activating" biomaterial. Based on theoretical considerations and experimental investigations of the physical mechanisms of the contact activation of proteins, it was shown that electron transfer processes between protein macromolecules and an implant's surface directly result in an activation of the proteins. Silicon carbide is an optimal biomaterial with electronic properties adapted to the physiologic environment. Protein activation, platelet adhesion and activation, as well as endothelialization are significantly improved when compared to 316L stainless steel. Thus, it was possible to show in-vitro, that key processes contributing to long-term complications such as restenosis of stents are inhibited with a-SiC:H. In this regard a-SiC:H can be described as a "smart material." Further improvements in the clinical outcome of interventional stent procedures are possible only when the same sound scientific methods leading to a detailed understanding of the various interaction processes between the implant and the surrounding biological environment are stringently applied.

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