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Facets of protein assembly on nanostructured titanium oxide surfaces

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ABSTRACT

One key for the successful integration of implants into the human body is the control of protein adsorption by adjusting the surface properties at different length scales. This is particularly important for titanium oxide, one of the most common biomedical interfaces. As for titania (TiO_2) the interface is largely defined by its crystal surface structure, it is crucial to understand how the surface crystallinity affects the structure, properties and function of protein layers mediating subsequent biological reactions. For rutile TiO_2 we demonstrate that the conformation and relative amount of human plasma fibrinogen (HPF) and the structure of adsorbed HPF layers depend on the crystal surface nanostructure by employing thermally etched multi-faceted TiO_2 surfaces. Thermal etching of polycrystalline TiO_2 facilitates a nanoscale crystal faceting and, thus, the creation of different surface nanostructures on a single specimen surface. Atomic force microscopy shows that HPF arranges into networks and thin globular layers on flat and irregular crystal grain surfaces, respectively. On a third, faceted category we observed an alternating conformation of HPF on neighboring facets. The bulk grain orientation obtained from electron backscatter diffraction and thermodynamic mechanisms of surface reconstruction during thermal etching suggest that the grain and facet surface-specific arrangement and relative amount of adsorbed proteins depend on the associated free crystal surface energy. The implications for potentially favorable TiO_2 crystal facets regarding the inflammatory response and hemostasis are discussed with a view to the advanced surface design of future implants.

1. Introduction

When a biomaterial comes into contact with body fluids proteins are adsorbed on the surface within a short period of time [1]. Their surface-induced arrangement and conformation [2] mediate biological reactions at the biointerface and subsequent cellular behavior [3].

Amongst various material surface properties surface chemistry and surface roughness have been recognized as major factors influencing implant-mediated host tissue responses and implant biocompatibility [4,5]. For example, titanium-based surfaces with a high free surface energy were reported to be more osteogenic than those with a low free surface energy [4]. Increasing surface roughness enhanced the attachment and proliferation of human bone marrow cells on titanium-based Ti6Al4V alloys [5]. In most cases enhanced cellular reaction was related to the protein adsorption behavior, with a crucial role being played by the structure and constitution of the adsorbed protein layers.

Titanium and its alloys belong to the most commonly applied implant materials with applications in the cardiovascular field, dentistry, and orthopedics [6,7]. Chemical surface treatments, coatings, or anodization of titanium to form TiO_2 are applied to increase the bioactivity of titanium-based implants [7–9].

To gain a comprehensive insight into how facet surfaces of titania (TiO_2) with different crystal orientations influence the adsorption of proteins we used thermal etching of polycrystalline TiO_2 to create molecularly self-assembled surfaces. We subsequently employed these defined TiO_2 surfaces to adsorb the human plasma protein fibrinogen (HPF), a key component in the blood coagulation cascade and, therefore, relevant to the onset of implant surface-induced blood coagulation and acute inflammatory responses [10].

HPF is an anisotropic, amphiphilic protein with a trinodular structure that consists of a hydrophobic E domain centered between two hydrophobic D domains, each of which is connected to an outer positively charged hydrophilic αC domain. The amount

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and assembly of HPF adsorbed on a surface are known to depend on the surface chemistry [11,12] and topography [13–15].

Even though it is now recognized that a nanoscale surface morphology can affect the adsorption of polypeptides and proteins, such as HPF [16–18], several apparently contradictory studies have documented that this does not generally seem to be the case. Whereas nanoscale surface roughness was observed not to influence the amount of HPF adsorbed on physical vapor deposited titanium films [19], the amount increased with increasing surface roughness on titanium created using a supersonic cluster beam which induced nanoscale pores [20]. These studies suggest that apart from surface chemistry and surface topography more subtle surface properties could affect the adsorption of proteins.

On flat hydrophobic surfaces HPF assembles in a trinodular shape and builds ring-like network structures indicating protein–protein interactions via their outer hydrophilic domains, whereas on hydrophilic surfaces HPF adopts a globular shape with the hydrophilic domains attached to the surface [21]. Recently we elucidated the role of crystallinity and how nanoscale lateral confinement can affect the assembly of adsorbed HPF on ultra-high molecular weight polyethylene (UHMWPE) surfaces. We observed that crystalline UHMWPE lamellae suppress the formation of ring-like HPF networks and that HPF molecules are preferentially adsorbed on crystalline lamellar sites of high free surface energy, thereby forming a densely packed protein layer [14,22]. Further systematic investigations of the influence of crystallinity and crystal orientation on the protein adsorption behavior are, however, sparse.

The approach followed in this work addresses these issues for the first time to the best of our knowledge as it permits clarification of the systematic influence of different TiO_2 crystal surfaces on protein adsorption behavior in a simultaneous adsorption experiment. Information on the crystal surface orientation was deduced from the bulk crystal orientation obtained by electron backscatter diffraction (EBSD) and taking into consideration thermodynamic mechanisms of surface reconstruction during thermal etching. The thermally etched TiO_2 grain surfaces were analyzed by atomic force microscopy (AFM) prior to and after the adsorption of HPF. Implications of the observed crystal facet-controlled, grain-specific adsorption of HPF on thermally etched TiO_2 surfaces on inflammatory reactions and hemostasis are discussed.

2. Materials and methods

2.1. TiO_2 surfaces

TiO_2 green bodies were produced by uniaxial compaction of TiO_2 anatase powder (Titandioxid Anatas Hombitan LW, Sachtleben Chemie GmbH, Duisburg, Germany) at 7.5 MPa followed by cold isostatic compaction at 500 MPa. The disk-shaped green bodies were sintered at 1460 °C for 16 h in air [23]. After cooling the surface of the TiO_2 specimens was mechanically ground with Buehler Ultra-Prep diamond grinding discs of 125, 70 and 45 μm abrasive size, and subsequently polished with Buehler MetaDi diamond suspensions of 15, 9, 6, 3 and 1 μm abrasive size (Buehler GmbH, Düsseldorf, Germany). Then the specimens were thermally etched at 1300 °C in air (heating rate 10 °C min^{-1}, maintained for 60 min) and after cooling to room temperature were stored for several hours in the ambient atmosphere before starting the protein adsorption experiment.

2.2. Protein adsorption

Phosphate-buffered saline (PBS) was prepared by dissolving 8.7 g NaCl, 1.82 g $K_2HPO_4 \cdot 3H_2O$ and 0.23 g KH_2PO_4 in 1 l of distilled water. Unless otherwise noted, all chemicals were purchased from Merck KGaA (Darmstadt, Germany). All buffer solutions were sterilized by autoclaving and filtered with a sterile 0.22 μm filter prior to use. Stock solutions of human plasma fibrinogen (HPF) (lyophilized from 20 mM sodium citrate–HCl, pH 4, Calbiochem/Merck Biosciences GmbH, Darmstadt, Germany) were dissolved in PBS at 37 °C to the stock concentration (200 μg ml^{-1}). Following reconstitution the aliquots were diluted with PBS at 37 °C to the final HPF concentration (10 μg ml^{-1}). The TiO_2 specimens were incubated in the HPF solution in PBS at 37 °C in a humidified atmosphere. The proteins were allowed to adsorb for 30 min under quasi-physiological conditions. After adsorption the TiO_2 specimens were rinsed twice with PBS and deionized water to prevent potential adsorption of residues from the buffer solution, and dried in a stream of nitrogen.

2.3. AFM analysis

Prior to and after the adsorption of proteins the TiO_2 specimens were analyzed by AFM (Dimension 3100, with Nanoscope IV controller, Digital Instruments, Santa Barbara, CA). Standard silicon cantilevers (Bruker AXS GmbH, Karlsruhe, Germany) with typical spring constants of 20–100 N m^{-1}, a length of 125 μm and a resonance frequency of 200–300 kHz were used in tapping mode in air at ambient temperature. The scan rate was 2 Hz. If not otherwise stated a first order plane fit correction was applied prior to the image analysis. Where possible the protein surface coverage was deduced by creating a binary image from the AFM height data and calculating the ratio of numbers of white and black pixels using standard software (ImageJ). Errors were obtained by varying the threshold values.

2.4. X-ray photoelectron spectroscopy (XPS) and contact angle analysis

Water contact angles were obtained from the shape of the axisymmetrical menisci of sessile distilled water drops using a DSA10 drop shape analysis system (Kruss GmbH, Hamburg, Germany). The surface chemistry was determined by XPS with a Quantum 2000 (PHI Co., Chanhassen, MN) apparatus with a focused monochromatic AlK_α source (1486.7 eV) for excitation. Spectra were fitted using the manufacturer's Multipak software. For the high resolution spectra the pass energy was set to 58.7 eV, with a spot size of 100 μm. Contact angles and XPS spectra were obtained from thermally etched TiO_2 specimens after immersion in PBS buffer for 30 min and subsequent drying to closely resemble the situation during protein adsorption.

2.5. Light microscopy

Light microscopic images of the TiO_2 specimen surface were taken with a Leica DMRXE (Leica Mikrosysteme GmbH, Wetzlar, Germany) in reflection mode with a Sony CCD camera DXC-930P (Sony Corp., Japan).

2.6. EBSD and field emission scanning electron microscopy (FE-SEM)

EBSD analysis was performed with an ESEM XL30 (FEI, Eindhoven, The Netherlands) operated with a LaB_6 cathode at 20 keV. The step size was 5 μm. The FE-SEM images were taken with an AURIGA 60 instrument (Carl Zeiss SMT, Oberkochen, Germany) at accelerating voltages of 0.5 and 2 keV at working distances of 1.3 and 11 mm, respectively. An in-lens detector was used for high resolution at 0.5 keV.