Physico-chemical properties of human plasma fibronectin binding to well characterized titanium dioxide

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Abstract

Biologically compatible implant materials are commonly used in medical applications. The physico-chemical nature of the implant surface influences macromolecular binding. In the case of titanium implants, these interactions are influenced by a thin titanium oxide layer (5–70 Å), the main oxide species is titanium dioxide (TiO₂). Shortly after implant insertion, a meshwork of fibrinogen and fibronectin forms between the soft tissue and the implant–oxide surface. Fibronectin is an important matrix glycoprotein that mediates the attachment of cells and proteins to each other or to the extra-cellular matrix. The objective of this study was to investigate the adsorption and desorption behavior of plasma fibronectin on titanium dioxide and measure the differences in the oxide particle surface potential caused by this binding. The surface of native titanium dioxide was characterized by X-ray diffraction, differential scanning calorimetry, and ESCA. Particle diameter was estimated by forward light scattering, and verified by scanning electron microscopy. Human plasma fibronectin radiolabeled with 125I was employed for adsorption and desorption studies on washed titanium dioxide samples. Electrokinetics were characterized by measuring the zeta potential (electrophoretic mobility) of titanium dioxide precoated with fibronectin.

Titanium dioxide exhibited different isoelectric values ranging from 5.2 to 6.4 as a function of pretreatment. Adsorption of fibronectin on washed titanium dioxide occurred within 10 min. with 57% binding. Desorption studies showed that fibronectin was not completely desorbed after a series of buffer washes over 69 h at pH 7.4. The adsorption isotherm had a plateau value of 0.21 mg m⁻². Zeta potential measurements demonstrated that the fibronectin coating caused an increase in the negative zeta potential. These findings give an insight into how titanium oxides might be altered to provide improved matrix glycoprotein adsorption and increased host cellular binding for enhanced implant biointegration. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Titanium dioxide; Fibronectin; Adsorption; Zeta potential

Introduction

Interaction of metallic implant surfaces with surrounding tissues and body fluids is a complex process involving different cell types and host factors. Upon surgical placement of an implant, the implant metallic surface and the surgically exposed tissue come into close apposition leading to various physico-chemical and biochemical interactions between the metallic surface and macromolecules from the soft tissue, as well as body fluids containing various macromolecules and dissolved inorganics. Some macromolecular species are adsorbed rapidly, some desorbed and replaced by other proteins, and still others denature or fragment once bound [1,2].
The physico-chemical nature of the implant metallic surface can be expected to influence macromolecular binding to it [1,2]. In the case of titanium, the most common metal used in medical and dental implants, this interaction is influenced by a thin 0.5–7 nm titanium oxide layer [3] which prevents corrosion and hydrogen embrittlement [4]. The main oxide species is TiO₂ [5]. This layer undergoes changes, becoming increasingly polarized and thicker upon prolonged exposure to a physiological environment [6,7]. Therefore, this oxide layer plays a major role in protein binding and cellular interaction.

It has been reported that shortly after implant insertion, a meshwork rich in fibrinogen and fibronectin forms between the soft tissue and the implant surface [8,9]. Fibronectin is a matrix glycoprotein that mediates the attachment of cells and proteins to each other, as well as to the extracellular matrices [10]. Fibronectin exists in a soluble form in plasma and as insoluble cellular forms in association with cell surfaces and extracellular matrix [11,12]. The plasma and cellular fibronectins are similar in structure and properties [13,14]. Plasma fibronectin is a disulfide-bonded dimer \( M_r = 440,000 \). It is composed of several structural domains that are linked by protease-sensitive peptide segments [15–18]. These domains have specificity of binding to cells, bacteria, collagen, glycosaminoglycans, actin, fibrin, fibrinogen, and other biological matter [10,16,19].

Because fibronectin is involved in cell adhesion and extracellular matrix interactions, its physicochemical behavior at implant surfaces is of particular interest. Fibronectin will bind to a wide variety of artificial substrates and promote cell adhesion to them [20,21]. It has the capacity to bind to both hydrophilic and hydrophobic surfaces [22–26]. Since the nature of protein binding may be affected by the physico-chemical characteristics of the oxide layer formed, a better understanding of this interaction is important in the development and modification of any implanted device. Therefore, the objective of this work was to investigate the adsorption and desorption mechanisms of fibronectin on well characterized TiO₂, and to determine changes in oxide particle interfacial potential as a result of its adsorption.

2. Materials and methods

2.1. Materials

2.1.1. Titania

Titanium (IV) dioxide (99.99% pure) was obtained from Aldrich Chemical (Milwaukee, WI). The loss on ignition of 0.055% and traces of calcium and iron (65 and 5 ppm respectively) were specified by the manufacturer.

2.1.2. Fibronectin

Human plasma fibronectin was obtained in lyophilized form from Gibco BRL (Life Technologies, Inc. Grand Island, N.Y.). The protein was reconstituted in phosphate buffered saline (PBS), pH 7.4 and stored at 4°C in plastic vials.

2.1.3. Reagents

PBS was obtained from Gibco BRL (Grand Island, NY). Sodium nitrate and sodium hydroxide (Fisher Scientific) were of ACS reagent grade.

2.2. Methods

Characterization of titanium dioxide powders was performed before and after fibronectin adsorption. The characterization methods used were: X-ray diffraction, size determination by light scattering, scanning electron microscopy, surface area by nitrogen adsorption, differential scanning calorimetry, and zeta potential by microelectrophoresis. The X-ray diffraction pattern (Phillips 3000 powder diffractometer, 35 kV, 25 mA) of the titanium dioxide was evaluated to determine the oxide allomorph present. Particle diameter was measured using forward light scattering (Microtrac Model 7995-10, Leeds and Northrup, Inc., St. Petersburg, FL) in dilute suspension and by scanning electron microscopy (Cambridge Stereoscan 250 MK2). The specific surface area was measured by Brunauer–Emmett–Teller (BET) nitrogen adsorption with a Quantasorb Sorption System (Quantachrome Corp., Boynton Beach, FL) in which the sample was first degassed at 300°C for 4 h. Differential scanning calorimetry was performed on the titanium powder with a DSC 910S TA Instrument (New Castle, DE) in which 17.77 mg titanium dioxide.
was equilibrated at 80°C and the temperature increased at a rate of 10°C/min to 600°C. Zeta potential measurements were performed using 0.01 \% of titanium dioxide in 0.03 mol dm\(^{-3}\) NaNO\(_3\) with a Pen Kem Lazer Zee Model 501 (Bedford Hills, NY). In order to remove any particle surface contaminants, several grams of titanium dioxide were washed in dilute NaOH solution (0.01 mol/dm\(^{-3}\)) using a continuous extraction Soxhlet apparatus, followed by three washings with Nanopure water (17.5 MΩ/cm). Washed samples were dried in a vacuum desiccator for 24 h at room temperature and stored in stoppered Pyrex glass containers to prevent atmospheric contamination. ESCA analyses of unwashed and washed titanium dioxide samples were conducted using a Perkin-Elmer Physical Electronics model 5600 ESCA spectrometer. Samples were probed to a depth of 100 Å. An analysis area of 800 μm\(^2\) was used for characterization of all samples.

2.3. Adsorption and desorption

Adsorption, desorption, and kinetic experiments were conducted with radiolabeled fibronectin. Fibronectin (0.05 mg) was incubated with 0.5 mCi Na\(^{125}\)I (New England Nuclear), oxidized with 250 mg/ml Chloramine T for 10 min, followed by a 96 mg/ml sodium metabisulfite reducing agent in 0.05 M sodium phosphate buffer (PBS) containing 1% BSA, pH 7.5. The iodinated matrix protein purification was accomplished on a Sephadex G50M column (Pharmacia), and the counts per minute (CPM) determined for the eluates on a 5236 Packard Auto-Gamma Spectrometer (Downers Groves, IL). The background CPM were obtained using control samples of fibronectin in PBS. To ensure that \(^{125}\)I labeling had no effect on the adsorption properties, labeled and unlabeled proteins were used in different concentration ratios [27]. Within experimental errors no preferential adsorption could be detected in these experiments. Purity of the labeled preparations was verified by comparing labeled with unlabeled protein by SDS-PAGE electrophoresis. To reduce the effect of adsorption of fibronectin onto the glass reaction vessels [28], polypropylene Eppendorf tubes and plastic pipette tips were used, and all containers were prewetted before adding the protein solution [28].

2.3.1. Adsorption kinetics

50 mg of titanium dioxide powder was suspended in 0.5 ml PBS in Eppendorf tubes and equilibrated for 1 h on a chemical rotator (Fisher Scientific) at 25°C. \(^{125}\)I labeled fibronectin (5 ng) in 0.5 ml PBS was added to the titanium dioxide powder. The samples were then mixed on a rotator (Scientific Industries, Inc., Bohemia, NY) at room temperature for times ranging from 1 min to 24 h, centrifuged at 5000 rpm, and the CPM measured on a 5236 Packard Auto-Gamma Spectrometer. The supernatant was removed and both the isolated supernatant and remaining contents of Eppendorf tubes were counted. To correct for adsorption to the tube walls, the Eppendorf tubes were washed three times with PBS to remove powder, and the tubes recounted. Binding to titanium dioxide was determined based on the \(^{125}\)I content of the supernatant and container at each time interval. The time intervals for adsorption were 1, 10, 30, 60 min, and 2, 8, and 24 h. Measurements were performed in triplicate for each time point.

2.3.2. Desorption

Samples were prepared in the same manner as for the adsorption assay. After 1 h of incubation with radiolabeled fibronectin, the supernatant was replaced by an equal volume of PBS buffer. At specific time intervals (15 min, 30 min, 8 h, 20 h, 45 h, 59 h, 69 h), the samples were centrifuged, and the supernatant replaced with an equal volume of buffer. To account for any interstitial fluid remaining between the titanium dioxide particles, the precipitate and Eppendorf tubes were weighed on a microgram balance at each time interval before and after supernatant removal. Again, all measurements were performed in triplicate.

2.3.3. Adsorption isotherm

The adsorption isotherm of human plasma fibronectin on the oxide was determined by suspending 50 mg titanium dioxide powder in 0.5 ml PBS and equilibrating for 1 h at room temperature using a tube rotator (Scientific Industries, Inc.)
Bohemia, NY). Each sample was then incubated with 0.5 ml of a solution containing fibronectin in PBS (0.0750 to 1.800 mg ml) and equilibrated for 1 h on the rotator at 25°C. The samples were centrifuged at 5000 rpm and the supernatant collected for protein analysis. The pH of the samples was measured as pH 7.4. Initial and residual concentrations of fibronectin in solution were measured using a total organic carbon analyzer (Dohrmann DC90). To determine the extent of protein binding to the polyethylene tubes, triplicate control samples (without TiO₂ powder) were run in parallel and analyzed using a BioRad protein assay (Bio-Rad Laboratories, Hercules, CA). The protein assay allowed the determination of any protein that may have adsorbed to the reaction vessel despite the careful protocols followed. Protein adsorption density, \( r \), was calculated from depletion values. The parking area per molecule was calculated from the adsorption plateau value and the particle surface area as determined by the BET technique and using a molecular weight of 440 000 [29] for the protein.

2.3.4. Electrokinetic studies

Zeta potential of both titanium dioxide powder and titanium dioxide powder reacted with HPF was determined by measuring the particle electrophoretic mobility using a PenKem Lazer Zee Model 501 (Bedford Hills, NY), pH 7.4.

3. Results

X-ray diffraction analysis showed the presence of only one crystalline TiO₂ phase, rutile. The mean TiO₂ particle diameter was 7.4 ± 3.3 nm by forward light scattering with a Microtrac System. Scanning electron microscopy also showed a large number of smaller particles (<1 μm) not registered by the Microtrac System (Fig. 1). The particle surface area was measured to be 2.3 m² g using the BET method.

A comparison of zeta potential of titanium dioxide at different pH values for both unwashed and washed uncoated samples with 0.01 mol dm⁻³ NaOH (Fig. 2) shows the unwashed titanium dioxide to exhibit two isoelectric points (5.2 and 6.4) while the washed showed one at 6.2. The unwashed sample was negative at values below pH 5 while the washed samples were positive.

Differential scanning calorimetry (DSC) detected no bulk contaminants at temperatures up to 600°C (results not shown). However, ESCA analysis of unwashed and washed titanium dioxide (survey spectra given in Fig. 3) showed washing to reduce the amounts of silica, organic nitrogen, and chloride species on the surface (Table 1).

Adsorption of radiolabeled fibronectin on titanium dioxide occurred rapidly with maximum binding in less than 10 min and a definite decrease at longer times (Fig. 4). The percentage adsorbed...
Fig. 3. ESCA survey spectra of titanium dioxide before (a) and after (b) washing.

Fig. 4. Kinetics of human plasma fibronectin adsorption on titanium dioxide (in PBS, pH 7.4) — mean ± SD. The y-axis shows the percentage of fibronectin adsorbed as a function of time.

is defined as that percentage of initial protein in solution which binds to the oxide particle under the experimental conditions. Desorption of fibronectin from the particles by a series of buffer washings over time showed that a significant reduction in adsorbed protein occurred during the initial buffer washings (Fig. 5) with minimal subsequent removal.

Evaluation of the adsorption isotherm of unlabelled fibronectin onto titanium dioxide showed an initial slope suggestive of a moderate affinity. The isotherm demonstrated a plateau value of 0.21 mg/m² (Fig. 6) and the plateau value for adsorption density corresponds to a fibronectin monolayer. From the adsorption plateau, the

Table 1

<table>
<thead>
<tr>
<th>Element</th>
<th>Concentration (at. %)</th>
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<tr>
<td>C₁₆</td>
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<tr>
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<td>0.31</td>
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<tr>
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</tbody>
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Fig. 5. Desorption of human plasma fibronectin from titanium dioxide by subsequent washing in PBS — mean ± SD. The y-axis shows the percentage of fibronectin remaining as a function of wash number.
Fig. 6. Adsorption isotherm of human plasma fibronectin on titanium dioxide (in PBS, pH 7.4) — mean ± SD.

Fig. 7. Adsorption isotherm of human plasma fibronectin onto titanium dioxide and corresponding particle zeta potential after adsorption (in PBS, pH 7.4) — mean ± SD.

calculated parking area per molecule was $3.48 \times 10^3 \text{ nm}^2$.

A comparison of the zeta potential to the adsorption isotherm shows a good correlation between the increase in adsorption density of fibronectin on titanium dioxide and the increase in negative zeta potential (Fig. 7).

4. Discussion

Results in this paper indicate that the initial fibronectin attachment to titanium dioxide occurs rapidly at physiological pH. Desorption occurs with repeated washings, but the adsorption process is not fully reversible. These results support the interpretations that fibronectin has a moderate affinity for titanium dioxide.

The surface of the powders used for the adsorption studies was, as previously reported [30,31], slightly anionic, and based on ESCA data, showed the presence of impurities of silica, organic nitrogen, and chloride species. These impurities can result in a shift in isoelectric point (iep) of the oxide [30]. The anionic contamination may be attributed to the presence of TiCl$_4$. It has been reported that titanium dioxides show iep values ranging from 4.6 to 6.4 due to variations in pretreatment heating, washing protocols, atmospheric interactions, and method of storage [31,32].

The binding of fibronectin to the titanium dioxide powder was comparable to that reported for its binding to germanium [33]. This is not surprising since germanium also has a hydrophilic surface similar to titanium dioxide [33–36] and since adsorption is determined in this case more by the surface of the adsorbate than by the nature of the adsorbent [37]. In this study only 57% of fibronectin adsorbed (Fig. 4), possibly due to the hydrophilic nature of the particle surface [38]. Fibronectin is known to adsorb to a greater extent on hydrophobic rather than hydrophilic surfaces [28,39]. Similar binding characteristics have been reported for other serum proteins on similar surfaces [40–43].

This study focussed on binding to well characterized titanium dioxide because the chemical nature of the solid surface determines the mode and strength of binding and conformational changes of the adsorbed protein [37]. Antibody binding to adsorbed fibronectin showed more exposure of antigenic sites when the protein is bound to hydrophilic than onto hydrophobic tissue culture plastic [44]. Such surface dependent conformational changes have been observed by ellipsometry [39,45] and total internal reflection fluorescence spectroscopy [46]. Heparin and cell binding fragments of fibronectin become more exposed after binding to pretreated heparinized and nonheparinized poly(vinyl chloride) surfaces, suggesting protein orientation to be surface and
precoating dependent [47]. Intersubunit distance of two Gln3 residues increases [48], two free sulfhydryl groups (SH1 and SH2) show positional changes [49,50], and two amino-terminal regions become separated upon surface binding to Cytodex beads [51], all verifying that fibronectin undergoes conformational changes during binding.

Another important factor in protein adsorption is related to the nature of the protein itself. Two homologous serum proteins from dissimilar species (bovine and human albumin) showed considerable adsorption differences when tested on a silica-titania surface [52]. These results show that the adsorption behavior of two serum proteins is greatly influenced by their respective amino acid sequences. Moreover, single point mutations have been shown to have a major impact on protein adsorption on a variety of Si(Ti)Ox surfaces with positive, negative, and neutral charged coatings [53].

The slight decrease in attachment of fibronectin to the titanium dioxide after 1 h (Fig. 4) may be related to protein–particle surface concentration. Protein unfolding, as observed with atomic force microscopy, decreases with increasing particle surface coverage [54-57]. This decrease in protein structural rearrangement and unfolding results in a reduction of potential exposed binding sites reducing surface adsorption [58-60].

Fibronectin on titanium dioxide showed a partial desorption profile (Fig. 5) similar to hydrophilic silica [39]. Protein adsorption has been described as a nonequilibrium irreversible process because many proteins do not completely desorb from metal oxide surfaces [2,45,60,61]. Some irreversibility may be due to the protein’s tendency to denature over period of time [62,63]. Specific binding activity is related to protein size due to multiple protein contacts on a surface [55]. Simultaneous rupture of most of these bonds at a greater number of sites would be required to totally dislodge the protein. Since fibronectin is a large molecule with numerous binding sites, this may explain why desorption was not completely reversible [42,64]. Surface properties should certainly influence protein desorption in the same manner as the influence adsorption. Fibronectin, for example, is more easily removed from hydrophilic than hydrophobic polystyrene [44,65]. The presence of a negative surface potential of titanium dioxide may also influence desorption during the washing cycles. Desorption studies of adsorbed cytochrome mutants (E15Q and E48Q) using buffer washes found protein preadsorbed to negatively charged surfaces (stearic acid) were fully reversible. Only on highly positive (polyallylamine) and neutral surfaces (POPC) was there minimal reversibility [53]. As in the case of adsorption, a small variation in amino acid composition can affect desorption [52].

The slope of the adsorption isotherm (Fig. 6) indicates a moderate affinity of the protein for the titanium dioxide surface [37]. Ellipsometric work on fibronectin binding to modified hydrophobic and hydrophilic silica has reported the binding to be 2.74 and 2.45 mg/ml respectively with a plateau value concentration of 20–30 µg/ml [39]. Differences between the values in the literature and our results could be due to the different substrates employed [65,66]. The surface concentration at the fibronectin isotherm was lower than the expected value based on molecular size. This has been observed in other protein–surface systems and can be explained by the formation of a less dense monolayer and unfolding of the molecule [37].

Zeta potential measurements showed the iep of the washed, hydrated titanium dioxide to be 6.2, and it has weakly negatively charged surface (−16 mV) under physiological conditions (Fig. 2). These values are in the range of previously published data [32,67,68]. The iep of fibronectin is 5.5–6.3 [13,69-71] and under the experimental conditions (pH 7.4) it would carry the overall negative charge as well. However, it is to be noted that different regions of the protein are markedly different from each other in terms of acidic and basic properties [70,72,73]. Hence, the protein adsorption process can be driven by the electrostatic interactions between the positive sites of the protein and the negative particle surface sites. The observed increase in negative zeta potential after protein adsorption (Fig. 7) can be accounted for by the tertiary geometric orientation the protein can assume during the process of protein binding. Fibronectin is known to assume different geometric
shapes depending on the particular surface it binds to and the conditions under which it has been preserved [33, 57, 74-77]. The degree with which conformational changes occur are also protein specific. Adsorbed Factor XII [78] and molecular IgG protein binding is reported to occur with extensive unfolding [79]. Circular dichroism [70] and ellipsometric studies [55, 80, 81] of fibrinogen, prothrombin, and serum albumin, show little or no rearrangement of the adsorbed layers. Aside from the different surfaces used, experimental conditions may also influence protein conformation. Plasma fibronectin in solution has been described as an extended structure under acidic, alkaline, or under conditions of high ionic strength when visualized by rotary shadowing [74, 75]. The use of 30% glycerol in the sample preparation of fibronectin for electron microscopic observation of rotary shadowed specimens is sufficient to yield extended structures [48, 76, 77]. In the absence of glycerol, the protein assumes a compact rounded structure.

5. Conclusions

Studies of plasma fibronectin binding to a well characterized implant material oxide have provided insights into the ways in which such materials might be manipulated to optimize their in vivo performance. Fibronectin was shown to bind rapidly to titanium dioxide with a moderate affinity. The measurement of fibronectin binding to titanium dioxide yielded an isotherm with a plateau value of 0.21 mg/m² at an equilibrium concentration of 0.4 mg/ml at pH 7.4. Fibronectin coating of oxide particles caused an increase in its negative zeta potential, partly as a result of electrostatic interaction between positively charged protein groups and the negatively charged particle exposing negatively charged protein groups at the fluid-protein interface. These results in a simple in vitro system suggest that by altering the implant oxide surface, it may be possible to manipulate protein binding in vivo. In doing this, by altering the properties of the bound protein such that cell binding domains are optimally presented, it may be possible to improve cell binding ability of implantable materials.

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