Protein Adsorption to Surface Chemistry and Crystal Structure Modification of Titanium Surfaces

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ABSTRACT

Objectives: To observe the early adsorption of extracellular matrix and blood plasma proteins to magnesium-incorporated titanium oxide surfaces, which has shown superior bone response in animal models.

Material and Methods: Commercially pure titanium discs were blasted with titanium dioxide (TiO_2) particles (control), and for the test group, TiO_2 blasted discs were further processed with a micro-arc oxidation method (test). Surface morphology was investigated by scanning electron microscopy, surface topography by optic interferometry, characterization by X-ray photoelectron spectroscopy (XPS), and by X-ray diffraction (XRD) analysis. The adsorption of 3 different proteins (fibronectin, albumin, and collagen type I) was investigated by an immunoblotting technique.

Results: The test surface showed a porous structure, whereas the control surface showed a typical TiO_2 blasted structure. XPS data revealed magnesium-incorporation to the anodic oxide film of the surface. There was no difference in surface roughness between the control and test surfaces. For the protein adsorption test, the amount of albumin was significantly higher on the control surface whereas the amount of fibronectin was significantly higher on the test surface. Although there was no significant difference, the test surface had a tendency to adsorb more collagen type I.

Conclusions: The magnesium-incorporated anodized surface showed significantly higher fibronectin adsorption and lower albumin adsorption than the blasted surface. These results may be one of the reasons for the excellent bone response previously observed in animal studies.

Keywords: titanium dioxide; magnesium; immunoblotting; fibronectins; albumins; collagen type I.

INTRODUCTION

In living systems, blood (plasma) is the first component to come in contact with biomaterials such as a titanium implant during surgery [1]. It is known that immediately after contact with plasma, rapid adsorption of plasma proteins onto the biomaterial takes place [2], which influences subsequent cell attachment, spreading, proliferation, and differentiation [3]. In bone-to-implant binding, i.e. osseointegration, the attachment of blood-derived proteins such as plasma fibronectin to the implant surface enhances the chemotaxis [4] and focal adhesion of osteogenic cells [5]. Fibronectin is a high-molecular weight extracellular matrix (ECM) protein (approximately 440 kDa) that binds to integrins, which are membrane-spanning receptor proteins [6]. One form of fibronectin, the plasma fibronectin, is produced by hepatocytes in the liver and circulates in the blood [7]. It activates signaling pathways that direct cell-cycle progression, gene expression, matrix mineralization [8], and the regulation of the survival of osteoblasts [9]. In addition, plasma fibronectin is known to be a regulator for bone density, and bone biomechanical properties [7], and it has been reported that plasma fibronectin interacts with bone morphogenetic protein 1, indicating that it has an important role in osteogenesis [10].

Ever since the importance of a moderately roughened surface was proposed, the rate of osseointegration has been enhanced by surface modification [11], and further, in recent times, surface modification is being carried out even at the nano-level. It is therefore reasonable that implant surfaces should be modified at this level, given that it has been proven that cells react sensitively to nano-topographies [12]. Several studies have investigated the effect of the modified surfaces on osteogenic cell reactions, and surface modifications aimed at enhancing cell responses have been carried out [13,14]. However, few modifications have actually focused on the protein reactions underlying such cell reactions.

We have used electrochemical oxidation incorporating protein "bindable" ions such as sulfate, phosphate [15], and calcium [16] to create surface modifications aimed to theoretically attract ECM proteins or bone matrix proteins with high bonding properties. The concept behind these novel modifications is the hypothetical biochemical bonding between bone and the implant [16]. These so-called "bioactive" implants showed improved bone responses compared to machined implants or other surfaces available on the market. A recent study by Sul et al. [17] validated the presence of a biochemical bond of surface chemistry modified smooth surface implants in bone but also measured the relative quantity of their biochemical bond strength at the bone-to-implant interface. Magnesium (Mg)-incorporated oxidized implants showed stronger and faster bone integration as compared to commercially available oxidized or dual acid-etched implants [18]. The enhanced bone response of the Mg implants was most likely due to the Mg titanate chemistry effects, possibly attracting ECM proteins or bone matrix proteins. It has been reported that Mg supplementation to young mice influenced bone formation, resorption, and mineralization [19]. It has also been shown that Mg deficiency may disturb bone metabolism and lead to osteoporosis [20].

The objective of this *in vitro* study was to investigate if adsorption of three different proteins, which are purportedly involved in bone apposition to implant surfaces is altered in Mg-modified surfaces, which would substantiate the *in vivo* data.

MATERIAL AND METHODS

Disc sample preparation

The disc samples (commercially pure titanium, CpTi, ASTM grade 4, 10 mm × 5 mm) were manufactured using a CNC (computer numerical control) machine and then blasted with TiO_2 particles in the range of 100 - 150 μm. The implants were degreased by sonication in an aqueous solution of phosphate-free Extran® MA 03 (Merck, Darmstadt, Germany)/deionized water (1 : 100) and absolute ethanol for 2 × 15 min. Next, they were rinsed with deionized water, and then dried in an oven at 60 °C for 24 h. The samples were divided into 2 groups, the blasted control group and the oxidized test group. The test group was fabricated in an electrolyte mixture containing Mg ions. They were fabricated using a micro arc oxidation (MAO) process as previously described [21,22]. In brief, the MAO process was conducted in galvanostatic mode, with the anodic forming voltage increased at a rate of dV/dt and controlled at 0.5 V/s by a combination of electrochemical parameters. The electrolyte mixture was stirred with a magnetic stirring bar at 300 rpm. Currents and voltages were recorded at 1-s intervals using a computer that was interfaced with the power supply. The samples were rinsed with deionized water and then dried in an oven at 60 °C for 24 h.

Surface properties and analysis techniques

The morphologies of the samples were observed using SEM (LV-SEM, JSM-6380LV; JEOL, Sollentuna, Sweden). The chemical composition of the samples was measured by X-ray photoelectron spectroscopy

(XPS, ESCALAB 250, Thermo-VG, England) using a monochromatic Al Kα X-ray source (1486.7 eV, 300 W; beam size, 400 μm diameter). The electron take-off angle was fixed at 45º and the vacuum pressure was maintained below 10^{-9} torr during spectral data acquisition. XPS data were acquired before and after sputtering. In order to remove the superficial contaminant (2 monolayers), Ar sputter cleaning was carried out for 3 s (beam energy, 2 KeV; primary current, 2 μA; raster area, 3.14 mm²). The binding energy of the target elements was determined with a resolution of 0.1 eV, using the binding energy of carbon (C 1s: 284.8 eV) as a reference.

The crystal structure was determined by low-angle XRD with a thin film collimator (X`Pert PRO-MRD, Philips Ltd, Netherlands) on a plate-type sample prepared with the same electrochemical parameters as the test screw-shaped implants. The step size used in the scan was 0.02° over the range of 15° to 70°. The spectra were recorded using Cu Kα radiation (0.154056 Å) generated at an acceleration voltage of 35 kV and a current of 25 mA.

Surface roughness was measured using an optical profilometer (MicroXamTM, Phase-Shift, Arizona, USA). Three discs each from the test group and from the control group were measured at 3 areas to give a total of 9 measurements for each group. The measuring area was 230 μm × 230 μm for each group. A Gaussian filter, 50 μm × 50 μm, was used to separate the roughness from errors of form and waviness.

Attachment of purified albumin to disc surfaces

Relative amounts of attached albumin was analyzed by electrophoresis and Coomassie blue staining of the gels after solubilization of the disc-associated albumin. Discs in triplicate were immersed in 40 mg/ml purified human serum albumin (Sigma, St Louis, MO, USA) for 16 h at 37 °C to saturate binding, then washed in phosphate-buffered saline (PBS). Attached albumin was solubilized by boiling discs for 5 min in detergent buffer (0.1% sodium dodecyl sulfate [SDS], 1% Igepal CA-630 and 0.5% sodium deoxycholate in PBS), and samples were analyzed by 5% SDS-polyarylamide gel electrophoresis followed by Coomassie blue staining of the gels. Evaluation of staining intensities was performed by analysing images using Sigma Gel software (SPSS Science Software GmbH, Erkrath, Germany).

Attachment of collagen and fibronectin to disc surfaces

A total of 100 μl with 0.2 mg/ml bovine plasma fibronectin (Sigma, St Louis, MO, USA) or bovine collagen type I (Nutacon BV, Leimuden, Netherlands) in PBS was added to the tops of the discs in triplicate and incubated for 16 h at 37 ºC. The discs were washed 3 times in PBS and transferred to microcentrifuge tubes containing 500 μl 1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% SDS. The discs were boiled in this solution for 5 min, then chilled on ice, and the solutions were stored at - 80 ºC. The samples were thawed on ice, and 25 μl of each sample was mixed with 25 μl 2XLaemmli sample buffer (BioRad, Hercules, CA, USA) and a final concentration of 8% β-mercaptoethanol. The samples were boiled for 5 min, chilled on ice, and run on a 5% SDS polyacrylamide gel electrophoresis. Proteins were electro-transferred to polyvinylidene difluoride membranes (BioRad), and plasma fibronectin and collagen type I were detected by immunoblotting with specific antibodies. Rabbit anti-human fibronectin antibodies F-3648 were obtained from Sigma (Sigma, St Louis, MO, USA), and rabbit anti-human collagen type I antibodies ab292 were from Abcam plc, Cambridge UK. Blots were developed using horseradish peroxidase-coupled secondary antibodies and an Advance Western Blotting Detection Kit (GE Health Care, Buckinghamshire, UK). Evaluation of staining intensities was performed by analysing images using Sigma Gel software.

Statistical analysis

All statistical analyses in the present study were performed with the KaleidaGraph software (Synergy Software, Essex Junction, VT, USA). The mean and standard deviation values for the *in vitro* parameters were calculated. The average values were compared by paired Student's t-test and analysis of variance (ANOVA) followed by a post hoc Tukey-Kramer test with the value of statistical significance set at the 0.05 level.

RESULTS

Surface characterization

Figure 1 shows SEM micrographs that characterize blasted pits and facets in the control surface and homogeneous porous structure with an average pore size of 1 - 2 μm in the test surface. The surface roughness after filtering showed an Sa value (arithmetic average height deviation, μm) of 0.81 (± 0.31) for the control and 0.75 (± 0.14) for the test group. No significant differences regarding surface roughness were observed. Figure 2 shows high resolution XPS spectra of the major elements extracted from the Ti 2p3/2
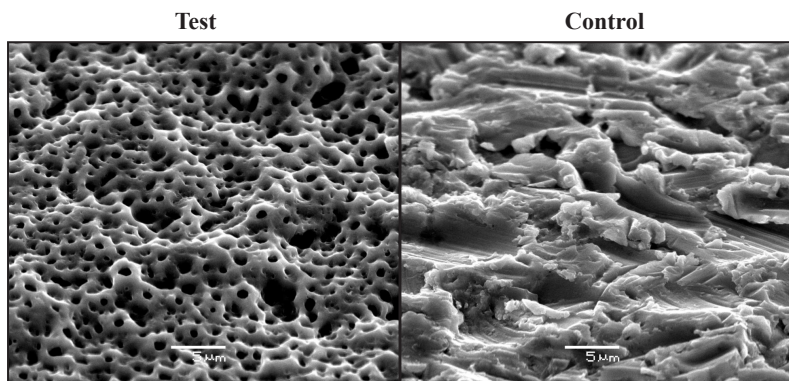
Figure 1. Scanning electron microscopy image of the surface blasted with titanium particles (control), and Mg-incorporated anodized surface (test) (Scale bar: 5 μm).

(458.9 ± 0.1 eV), O 1 s (531 ± 0.5 eV) (Figure 2A), and Mg 2p (50.4 ± 0.1 eV) core-level energy regions of the electron orbitals before and after argon ion (Ar+) sputter cleaning (Figure 2B). Table 1 shows the quantitative differences between the chemical compositions of the samples. The test sample showed the major doublet peaks of the O 1 s at 530.8 eV and 531.7 eV, which may be attributed to the Mg titanate and –OH functional groups. The blasted implants consisted mainly of TiO_2. Figure 3 shows the XRD patterns of the amorphous structure in the control group and a mixture of anatase and rutile phase in the test group (Figure 3).
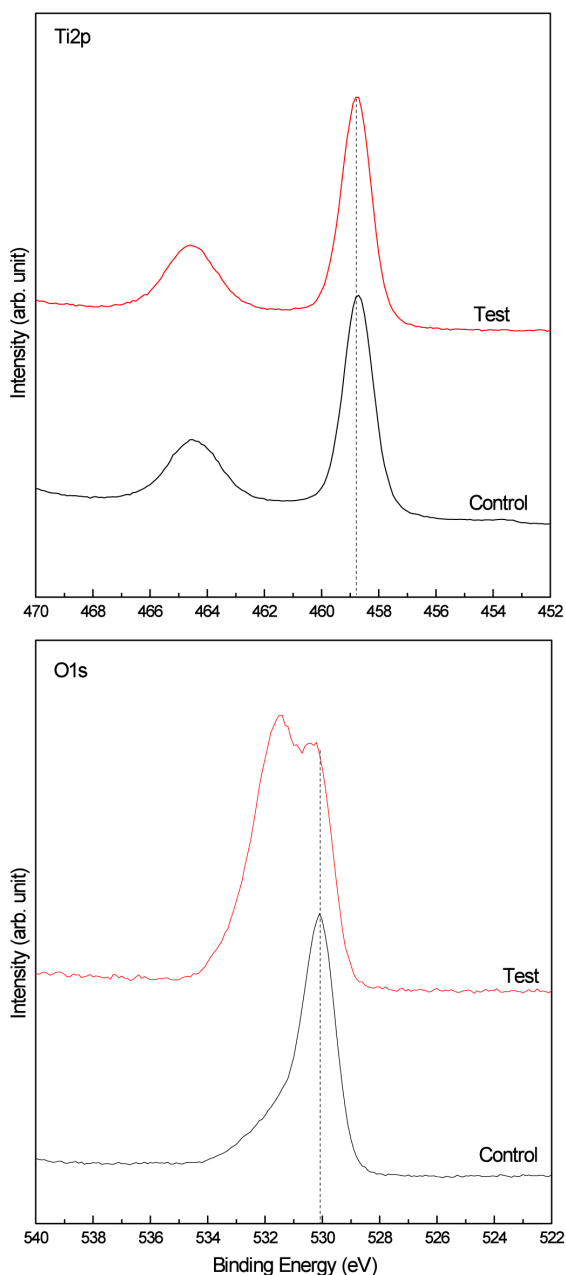

Figure 2B. Mg cation incorporation during the MAO process, characterizing the binding energy at the Mg 2p of as-received and Ar + sputter-cleaned surfaces. The dashed line indicates the binding energy of peak position at Mg 2p of as-received surface.

Figure 2A. Ti 2p and O 1 s spectra of control and test surface. The dashed line indicates the binding energy of peak position at Ti 2p and O 1 s for the control surface.
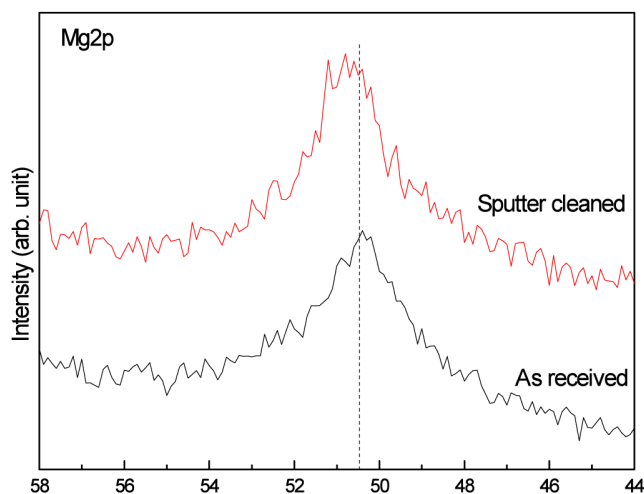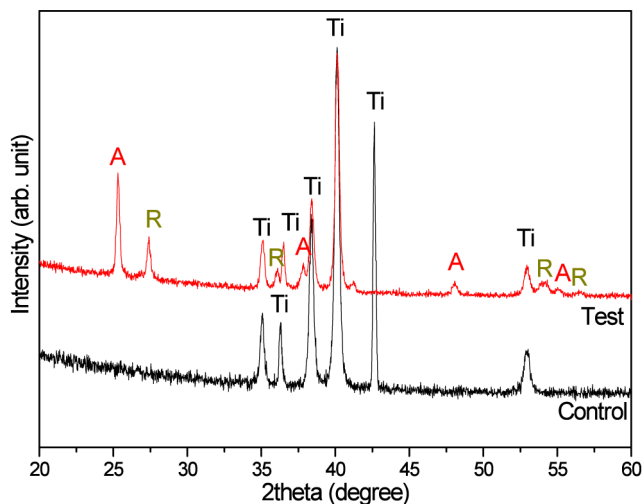
Figure 3. X-ray diffraction patterns of control and test surface. Amorphous, anatase and rutile phase of TiO_2 were detected on the control and test groups. Ti = titanium; A = anatase; R = rutile.

Table 1. Binding energies[a] and atom concentration rate[b] of elements at as-received and sputter cleaned surface in XPS analysis.

Atom	Control				Test			
	Before[c]		After[d]		Before[c]		After[d]	
	at.%	BE	at.%	BE	at.%	BE	at.%	BE
Ti	12.5	458.8	19.3	458.8	10.0	458.8	17.9	458.9
O	55.8	530.2	67.9	530.3	53.1	531.5	60.7	530.5
Mg	-	-	-	-	6.9	50.4	9.2	50.5
C	31.3	284.8	12.8	284.8	27.9	284.8	9.8	284.8
N	0.4	400.2	-	-	2.1	400.3	1.6	400.4

[a]Binding energy value in eV.
[b]Atom concentration rate in at.%.
[c]As-received surface.
[d]Sputter cleaned surface.
BE = binding energies.

Adsorption of purified albumin, fibronectin, and collagen type I to control and test surfaces

The intensity of the protein stainings for each surface is shown in Figure 4A. The results of this study showed that significantly more albumin adhered to control surfaces than to test surfaces, and significantly less purified fibronectin adhered to control surfaces than to test surfaces ($P \leq 0.05$) (Figure 4B and 4C). For collagen type I, no significant difference was detected, although a binding tendency was found in favour of the test surface ($P = 0.09$) (Figure 4D).

DISCUSSION

This study focused on the effect of titanium surface property changes with particular attention to the initial protein behaviour. Surface characterization determined major differences of surface chemistry and crystal structure but minor differences of surface roughness between the control and test surfaces.

In the test group, the characteristic element of Mg, 7 - 9 at.%, was incorporated into the oxide layer through the field-associated ion incorporation during the MAO process [22,23]. The finding of the hydroxyl group in the test surface is consistent with the findings of previous studies [22,24].

It has been reported that enhanced osteogenic cell responses *in vitro*, and bone apposition *in vivo*, have been observed in surfaces possessing an external layer of anatase and rutile phases [25]. The present results of surface characterization are congruent with those of the implants used for the previous *in vivo* studies. This was an important aspect of our study, since its aim was to validate *in vivo* initial protein interactions with *in vitro* data, possibly correlating the latter to the enhanced bone
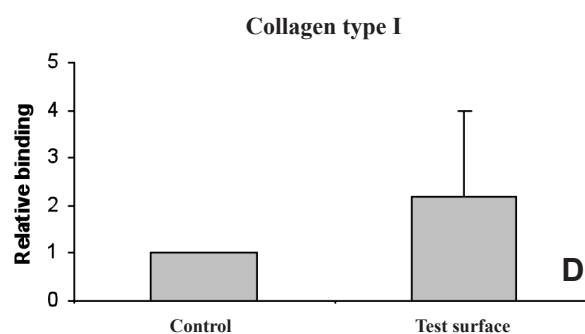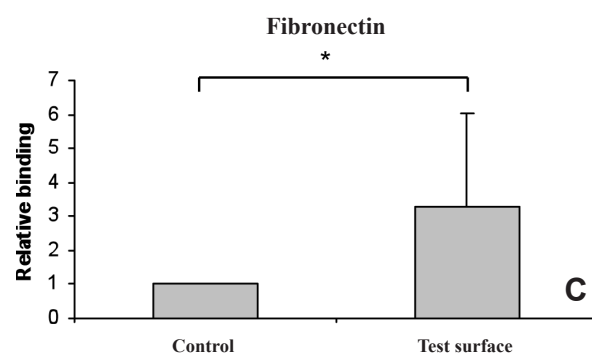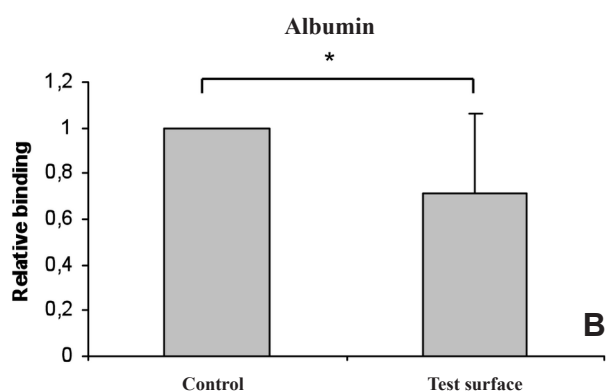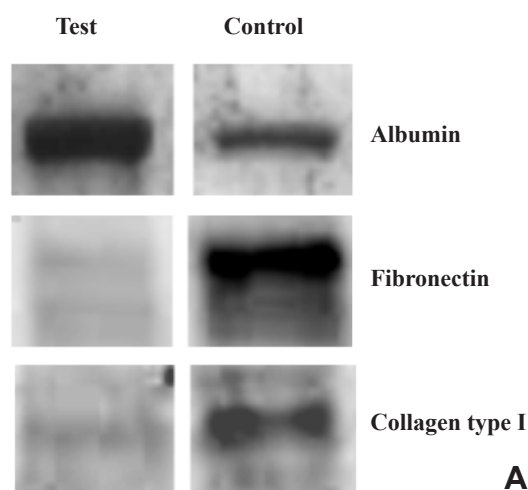
Figure 4. Attachment of extracellular matrix and blood plasma proteins to control and test surfaces (n = 3 in each group, performed in triplicate). Purified albumin, fibronectin, or collagen type I were allowed to attach to surfaces for 16 h at 37 ˚C. Relative binding was measured by electrophoresis followed by Coomassie blue staining or immunoblotting of solubilized proteins (A). Significant differences by densitometry (B - D) using paired Student's t-test ($P \leq 0.05$) are indicated (*).

apposition seen in the animal studies [21,22,24,26]. The individual protein adsorption test showed that the amount of albumin adsorbed onto the test surface was significantly lower than that adsorbed onto the control surface. Moreover, the amount of fibronectin adsorbed to the test surface was significantly higher than that adsorbed to the control surface. The amount of collagen type I adsorbed onto the test surface was also higher, although the difference was not statistically significant. The results of the study clearly showed the characteristics of each surface with regard to specific protein binding. It has been reported that osteoblasts grown on Mg-incorporated surfaces show higher expression of β1, and α5β1 integrin receptors than do non-Mg-incorporated surfaces [27]. Since the β1, and α5β1 integrin receptors are known to be fibronectin receptors, these results suggest that Mg attracts more fibronectin to the surface than occurs with non-Mg-incorporated surfaces.

In a preliminary experiment, we used human plasma obtained from healthy blood donors and incubated this on control and test samples for 16 h. The precipitated proteins were run on SDS-polyacrylamide gel-electrophoresis, and protein bands detected by Commassie blue staining were cut out of the gel and sent for protein identification by mass spectrometry (Pick'n Post Service, VWR International AB, Stockholm, Sweden). The results showed that at 16 h, the proteins above detection level were plasma albumin, and the amount of adsorption showed different results than that from purified albumin. Similarly, analysis of fibronectin by immunoblotting after incubation of human plasma on discs showed different results compared to corresponding purified protein demonstrated in the current study. It would be interesting to study more thoroughly the protein adsorption using human plasma. However, this is a difficult task because the amount and type of protein adsorption changes rapidly due to competitive protein adsorption [28] (Vroman effect). The Vroman effect is the competitive nature of protein adsorption onto the surface depending on the molecular weight of the protein [29]. In future studies, it will be interesting to observe different time points and clarify the mechanisms of this phenomenon.

Collagen type I is the major constituent of bone matrix protein [30], which is assembled in the presence of plasma fibronectin [31]. It is an essential protein in osteogenesis [32], which occurs later in the biological process. The reason for observing collagen type I adsorption in the individual protein adsorption test was to investigate its reaction to the Mg surface, because of its central role as structural component in bone, and the lack or abnormality of both collagen type I and Mg causes osteogenesis imperfecta [33].

Although there was no significant difference, the test surface tended to have higher amounts of collagen type I adsorption, which may be one of the factors for the enhanced bone apposition seen in animal studies.

Albumin is a major protein included in plasma (approx. 60%, molecular weight 65 kD) which is also a well-known blocking protein used in laboratory experiments. It has been reported that albumin has characteristics that prevent other protein adsorption and cell adhesion on its coated surface [34]. The relationship between plasma fibronectin and albumin has been investigated by Grainger and colleagues [35], who stated that albumin "masks" adsorbed plasma fibronectin and lowers the amount of cell attachment, and that on specific hydrophobic surfaces, albumin out-competes with other ECM proteins, including plasma fibronectin, even if the concentration of the plasma fibronectin is comparatively high. It is well known that plasma fibronectin binds more to hydrophilic surfaces [36], whereas albumin binds more to hydrophobic surfaces [37]. Anodic oxidized Ti surfaces have been reported to present hydrophilicity [38,39]. It has also been reported that anodic oxidized Ti surfaces have high surface energy [40,41], which is essential for maintaining surface hydrophilicity [42]. This suggests that the enhanced adsorption of fibronectin and reduction of albumin may be a result of surface energy-related hydrophilicity as well as Mg incorporation. Since surface roughness showed no significant differences, our study results strongly suggest the involvement of theses abovementioned factors.

CONCLUSIONS

In this study, the effect of titanium property changes on the amount of fibronectin, albumin, and collagen type I adsorption was investigated. Mg-incorporated titanium oxide surfaces showed major differences of surface chemistry and crystal structure, albeit similar surface roughness values compared to the control TiO_2 blasted surface. In the protein adsorption investigation, the test surface significantly reduced the adsorption of albumin and significantly enhanced fibronectin adsorption as compared to the control. The presence of Mg, the high surface energy, and hydrophilicity most likely influenced the enhancement of protein adsorption. This may be a reason for the enhanced bone apposition observed in previous animal studies.

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