Titanium implant surface modification is a key strategy used to enhance osseointegration. Platelets are the first cells that interact with the implant surface whereupon they release a wide array of proteins that influence the subsequent healing process. This study therefore investigated the effect of titanium surface modification on the attachment and activation of human platelets. The surface characteristics of three titanium surfaces: smooth (SMO), micro-rough (SLA) and hydrophilic micro-rough (SLActive) and the subsequent attachment and activation of platelets following exposure to these surfaces were determined. The SLActive surface showed the presence of significant nanoscale topographical features. While attached platelets appeared to be morphologically similar, significantly fewer platelets attached to the SLActive surface compared to both the SMO and SLA surfaces. The SLActive surface however induced the release of the higher levels of chemokines β-thromboglobulin and platelet factor 4 from platelets. This study shows that titanium surface topography and chemistry have a significant effect on platelet activation and chemokine release.

Keywords: Titanium, Hydrophilic, Platelet, Chemokine

INTRODUCTION

The clinical success of dental implant therapy relies on the process of osseointegration, whereby the unique biocompatible properties of titanium facilitates the direct apposition of newly formed bone onto the titanium implant surface. Surface micro-roughness, such as that obtained in sand blasted and acid etched (SLA) implants, has been well recognized to promote osseointegration when compared with smoother surfaces5. Further modification of the SLA surface leading to increased hydrophilicity (SLActive), has been shown in recent human in vivo studies to be associated with even further acceleration of the onset of osseointegration2,3. However, the underlying biological mechanisms responsible for these observations have not been fully elucidated. In vivo genomic analysis of gene expression during osseointegration4 and bone regeneration5 shows that these are complex biological processes involving the interaction of multiple mechanisms including inflammation, angiogenesis and osteogenesis. It has also been shown that these biological mechanisms can be influenced by titanium implant surface modification such as those seen with the SLA and SLActive surfaces5,7. Further, in vitro studies have shown that the SLActive surface is able to modulate the function of a number of cell types that are important in the healing process such as platelets8, undifferentiated mesenchymal cells9, osteoblasts10, macrophages11,12 and dendritic cells13.

As prominent blood components, platelets are one of the first cell types to reach the implant site whereupon they release a wide array of proteins that have widely recognized effects on the healing process14. While some of the secreted proteins such as fibrinogen, fibronectin and vitronectin have well known direct effects on bone healing e.g. by participating in thrombus growth, cell contact interactions, extracellular matrix composition and angiogenesis, many others, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and various other cytokines and chemokines can also modulate the response and behavior of other cell types involved in the healing process such as macrophages10, B cells13 and bone marrow cells. The platelet-derived chemokines; platelet factor 4 (PF4) and β-thromboglobulin (β-TG) are used as markers of platelet activation in this study. These chemokines are major components of the platelet α-granule secretome and play various differing roles in the healing process. Both chemokines have been shown to be involved in the autocrine regulation of megakaryocytopoiesis16, the chemoattraction (to varying degrees) of fibroblasts and early immune response mediators such as neutrophils and monocytes20 and the up-regulation of angiogenesis and vascular repair21.

Previous work has shown that increasing titanium surface topographical complexity can increase both platelet attachment and activation22,23. It is therefore reasonable to hypothesize that the early onset of osseointegration observed in vivo associated with topographically and chemically modified titanium surfaces may in part, be due to the influence of the titanium surface on platelet attachment and activation which in turn modulates the ensuing inflammatory response, bone wound healing process and ultimately, osseointegration. This study therefore aims to investigate the effect of these titanium surface modifications on the release of PF4 and β-TG from primary human platelets in vitro.
MATERIALS AND METHODS

Titanium
Grade II commercially pure titanium discs (1 mm thick, 15 mm diameter) with a smooth polished (SMO), large grit sand-blasted and acid-etched micro-rough (SLA®) or hydrophilic-modified SLA (SLActive®) surface were used in this study. The surface preparation of the SLA and SLActive discs has been described previously by our group and results in a microrough surface (Ra ~ 1.8 μm) identical to the dental implant surface currently used in clinical practice. Briefly, SLA (Sand blasted, Large grit, Acid etched) discs are produced by sand blasting titanium (corundum) with large grits (particle size 250–500 μm), followed by acid etching in a boiling mixture of hydrochloric and sulphuric acid. The discs are then cleaned in nitric acid, rinsed in deionised water, air dried and stored in aluminum foil. SLActive discs undergo the same sand blasting and acid etching process as for SLA. The cleaning, rinsing and storage steps all take place under the cover of nitrogen cover gas to prevent exposure to air. Final storage is in 0.9% NaCl solution at pH 4–6. The smooth titanium surface (Ra ~0.5 μm) was produced by polishing the discs (corundum) to a mirror finish. All titanium discs were kindly provided by the manufacturer Institut Straumann AG, Basel, Switzerland.

The relative surface wettability of the three surfaces was assessed qualitatively by examining the contact angle made by 50 μL of distilled water placed onto the titanium surface for 1 min. The surface topography of the titanium discs was analyzed using a scanning electron microscope (SEM; JSM-6610, Joel, NSW, Australia). The surface chemical composition was also analyzed with X-ray photoelectron spectroscopy (FEI Quanta 200 Environmental SEM with EDAX thin-window X-ray detector and microanalysis system).

Platelets
Ethical clearance was obtained from the Griffith University human research ethics committee to extract blood from human volunteers. Venous whole blood was subsequently drawn from three medication-free healthy volunteers without any relevant hematological medical history. The blood was collected in 4.5 mL CTAD (Sodium Citrate, Theophylline, Adenosine & Dipyridamole) buffered tubes (Becton Dickinson, Australia) and platelet rich plasma (PRP) was prepared following centrifugation at 160 g for 10 min. The PRP was aspirated and placed into a new tube ready for the in vitro experiments. Platelets poor plasma (PPP) was also prepared by centrifuging the PRP at 2,500 g for 20 min. Platelet concentration in the PPP was adjusted to 3×10^5 platelets/μL by diluting with the PPP. 600 μL of PRP from each volunteer was then cultured on each of the titanium surfaces in triplicate at 37°C in a 5% CO_2 atmosphere for one hour.

Scanning electron microscopy
Platelet morphology following attachment to the titanium surface was assessed by SEM. Briefly; attached cells were fixed overnight in 2.5% glutaraldehyde at 4°C. Following gradual ethanol dehydration and critical point drying (Autosamdi-815, Tousimis Research Corporation, USA), the samples were coated with platinum using a Bal-tec MED 020 Sputter Coater and analyzed by SEM.

Platelet attachment
The numbers of platelets attached to the titanium discs were assessed by photospectrometric measurement of lactate dehydrogenase (LDH) activity after the lysis of adherent platelets. The culture wells were first washed with saline three times to remove any platelets that did not adhere to the titanium discs. 200 μL of 1% triton buffer (Triton X-100, Sigma-Aldrich, Australia) was added to each well to lyse the adherent platelets. The culture wells were then incubated for 3 h at room temperature. After the incubation, 150 μL of each well was collected and analyzed for LDH activity using a cell cytotoxicity assay kit (LDH, AnaSpec, San Jose, CA). A standard calibration curve was obtained by measuring LDH release from known numbers of platelets lysed using the same protocol.

Platelet activation
β-thromboglobulin (β-TG) and platelet factor 4 (PF4) are proteins present within the platelet α-granules that are released upon platelet activation. Although many of the proteins released by the α-granules are also produced by other cell types, β-TG and PF4 are platelet specific and therefore, their presence in the culture supernatant can be used as a measure of platelet activation. An enzyme-linked immunosorbent assay (Asserachroms, Diagnostica Stago, Australia) was used as per the manufacturer’s instructions to quantitate the levels of β-TG and PF4 released into the culture medium following incubation of the platelets on the titanium surfaces for one hour at 37°C in a 5% CO_2 atmosphere.

Statistical analysis
The significance of any differences in platelet LDH, β-TG and PF4 concentration following culture on the different titanium surfaces was carried out using an analysis of variance (ANOVA) followed by a Fisher’s least significant difference test.

RESULTS
Surface analysis
The SLActive surface is obtained by rinsing/conditioning the hydrophobic SLA surface under a nitrogen atmosphere followed by storage in an isotonic sodium chloride (NaCl) solution. This procedure has been shown to increase the surface hydrophilicity and provides a cleaner (reduced surface carbon concentration) surface by reducing the adsorption of air-borne contaminants. The hydrophilic nature of each titanium surface is illustrated in Fig. 1 where the image shows a significant relative reduction in contact angle and hence increased...
hydrophilicity of the SLActive surface when compared with both the SMO and SLA surfaces. Although the contact angle was not specifically quantified, previous studies using the same surface preparations have shown that the hydrocarbon surface contamination which occurs within seconds of exposure to air decreases the surface free energy which is subsequently reflected by an increase in contact angle. i.e SLA (~130º) > SMO (~90º) >> SLActive (0º)\(^{25,26}\).

SEM analysis of the titanium discs before seeding with the platelets also confirmed that both the SLA and SLActive surfaces have, as expected given the same manufacturing process, similar micro-level surface topography (Figs. 2a,b and c,d). At the nano-scale level however, the SLActive surface also showed the presence of numerous discrete 20–50 nm sized, irregular shaped TiO\(_2\) structures on the surface (Figs. 2e,f). Other crystalline-like deposits noticed on the SLActive surface (Figs. 2c,d) were suggested by XPS analysis (Fig. 3) to be

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**Fig. 1** Illustration of the relative hydrophilicity (wettability) of the three implant surfaces. From left to right: SLActive, SLA, SMO.

**Fig. 2** Scanning electron microscopy analysis of SLA (A&B) and SLActive (C&D) surfaces at ×5K and ×30–40K magnification respectively. Arrows indicate deposits on the SLActive surface presumed to be from the storage solution, isotonic sodium chloride. High-resolution and magnification (×150–250K) of the SLActive surface (E&F) clearly shows nanoscale features present on the surface.
residual salt crystals from the isotonic storage solution.

Microscopic analysis of the platelets attached to the titanium surfaces showed the platelets acquired a spherical morphology with the presence of pseudopodia resulting in an overall stellate shape (Fig. 4) on all three (SMO, SLA and SLActive) implant surfaces.

Platelet attachment and activation
Following one hour incubation on the titanium discs, 1.3×10⁵, 2.5×10⁵ and 6.3×10⁴ platelets were shown to be attached to the SMO, SLA and SLActive surfaces respectively (Fig. 5) as measured by lactate dehydrogenase concentration. The number of platelets attached to the SLActive surface was significantly lower compared to both SMO and SLA surfaces (p<0.05).

No significant difference in the absolute concentrations either β-TG or PF4 released into the culture media following platelet activation was observed. However when these levels were corrected for the number of platelets attached to each surface, platelet activation by the SLActive surface was shown to achieve significantly higher levels (p<0.05) of both β-TG and PF4 secretion per attached platelet compared to both the SLA and SMO surfaces (Fig. 6). In contrast, the SLA surface was demonstrated to result in significantly
Numbers of platelets attached to the titanium surfaces following 1-h incubation at 37ºC in a 5% CO₂ atmosphere. Significantly higher numbers were attached to both the smooth (SMO) and SLA surfaces when compared to that seen on the SLActive surface (*p<0.01).

Surface roughness alone (SLA c.f. SMO) was shown to significantly decrease the release of both β-TG (♯p=0.014) and PF4 (*p=0.01). The addition of surface hydrophilicity however (SLActive c.f. SMO) was subsequently demonstrated to stimulate the release of β-TG (♯p=0.017) and PF4 (*p=0.007).

(p<0.05) lower levels of both β-TG and PF4 secretion per platelet compared to both the SMO and SLActive surfaces (Fig. 6).
chemokines have well described roles in the initiation of the subsequent inflammatory response. PF4 for example is chemotactic for inflammatory cells such as neutrophils and monocytes and can stimulate the activation and degranulation of neutrophils. Similarly, β-TG acts as a strong chemoattractant for fibroblasts and is weakly chemotactic for neutrophils. It can also stimulate mitogenesis, extracellular matrix synthesis, glucose metabolism, and plasminogen activator synthesis in cultures of human fibroblasts.

As the inflammatory response around implant materials has been clearly identified as an important factor in the healing process, the modulation of materials has been clearly identified as an important factor in the healing process. The formation of thrombin like structures in platelets vary significantly depending on the biomaterial surface, with the greatest spreading seen on the more hydrophilic surfaces. It is worth noting however that the titanium discs used by these researchers were coated using an ion-plating process to produce a range of discs with reduced surface free energy and therefore hydrophilicity. As our samples were uncoated, this may explain why no differences in cell spreading were noted, although our examination was qualitative only to indicate similar levels of platelet activation, with no attempt made to quantify the degree of cell spreading.

Titanium surface topography has been shown to significantly affect cells intimately involved in osteogenesis, bone wound healing and osseointegration. At the micro-scale, in vitro studies have shown that surface roughness enhances osteoblast differentiation and stimulates the local production of both osteogenic (TGF) and angiogenic (VEGF, FGF, EGF) growth factors. Moreover, in vivo studies have shown that a microrough titanium dental implant surface topography increases bone-to-implant contact and improves the clinical rate of wound healing.

As noted in this study, high magnification images of the SLActive surface also show the presence of discrete nanoscale sized features. Studies by others have demonstrated these nanostructures are indeed part of the active TiO2 surface layer and not crystallized NaCl. These nanoscale modifications have been shown to provide even further additional benefits in terms of osseointegration compared to micro-rough surfaces as measured by increased bone-implant contact, especially during the early stages of healing.

Whilst the SLA and SLActive surfaces have the same underlying micro-topography, it is not possible to determine at present (based on this study) whether the enhanced platelet activation we observed is a result of the hydrophilicity, nano-scale topography or some combination of these factors.

The activation and resultant differential secretion of proteins in response to different environmental stimuli, is currently a focus of research with emerging evidence showing that platelets may selectively release their stored proteins upon activation. In this study, we cultured the platelets on the titanium surfaces for one hour to enable complete activation and degranulation, and hence any selective temporal protein release was not a focus of this study. However, the possibility that titanium surfaces induce a topographic and/or chemistry specific early platelet protein release profile, as suggested by a recent report, is intriguing and warrants further investigation.

The present study showed that the secretion of platelet specific β-thromboglobulin and platelet factor-4 per platelet was significantly higher in response to the hydrophilic SLActive surface compared to its hydrophobic SLA counterpart after one hour of exposure. Similar results have also been demonstrated for the release of vascular endothelial growth factor-A and platelet derived growth factor, which further supports the hypothesis that titanium surface hydrophilicity enhances platelet activation and protein secretion.
However to paraphrase Goodman (1999)\(^9\), ‘no single measure is sufficient to describe the platelet response to biomaterials, but these measures can then be used to guide the interpretation of in vivo results. In this context, in vivo studies have shown that the SLActive surface is associated with an earlier onset of osseointegration\(^4,5\). As the biological processes of inflammation and bone formation have been shown to be inversely related during osseointegration\(^5\) it can be reasonably suggested that the higher platelet activation associated with this surface could contribute to minimizing the inflammatory period through the enhanced recruitment and degranulation of neutrophils which would in turn speed up the onset of the healing/osteogenic phase.

CONCLUSION

The present in vitro study shows that a nanotopographical hydrophilic titanium surface enhances platelet activation. This may represent a biological mechanism responsible for the more rapid onset of osseointegration observed clinically in vivo with the SLActive titanium surface.

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REFERENCES


