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ORIGINAL PAPER

Staphylococcus aureus adhesion to standard micro-rough and electropolished implant materials

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Abstract Implant-associated infections can cause serious complications including osteomyelitis and soft tissue damage, and are a great problem due to the emergence of antibiotic resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA). In some cases, antibiotic-loaded beads which release the antibiotic locally have been used, however such systems may lead to the development of antibiotic-resistant bacteria, as seen with gentamicin-loaded beads. Hence modifying the actual metal implant surface to inhibit or reduce initial bacterial adhesion may be an alternative option. This study describes the visualisation and quantification of *S. aureus* adhering to standard micro-rough 'commercially pure' titanium (TS) and Ti-6Al-7Nb (NS) surfaces, electropolished titanium (TE) and Ti-6Al-7Nb (NE) surfaces, and standard electropolished stainless steel (SS). Qualitative and quantitative results of *S. aureus* on the different surfaces correlated with each other, and showed significantly more live bacteria on NS than on the other surfaces, whilst there was no significant difference between the amount of bacteria on TS, TE, NE and SS surfaces. The results showed a significant decrease in the amount of bacteria adhering to the NE compared to standard NS surfaces. Such an observation suggests that the NS surface encouraged *S. aureus* adhesion, and could lead to higher infection rates in vivo. Hence electropolishing Ti-6Al-7Nb surfaces could be advantageous in osteosynthesis areas in minimising bacterial adhesion and lowering the rate of infection.

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Introduction

Osteosynthesis implant surfaces are generally designed to encourage soft and hard tissue adherence, eventually leading to tissue integration and osseointegration. Soft tissue infections and osteomyelitis are serious complications associated with implants, particularly open fracture injuries [1]. The ability of *S. aureus* in adhering to the extracellular matrix (ECM) and plasma proteins deposited on biomaterials, eventually forming biofilms, are significant factors in the pathogenesis of implant-associated infections [2–4]

Titanium, titanium alloys and stainless steel are commonly used for osteosynthesis implants, and the differences between stainless steel and titanium, and their biocompatibility are well documented [5–7]. Stainless steel implants are associated with significantly greater infection rates than standard titanium implants [5, 8]. A possible explanation for this is the fact that soft tissue adheres firmly to titanium implant surfaces [7, 9], whilst stainless steel implants encourage the formation of a fibrous capsule, enclosing a non-vascularised liquid filled void [9, 10]. In theory, bacteria can spread and multiply freely in this space, particularly as

it is less accessible to the host immune system. Recently, the adhesion and proliferation of fibroblasts was shown to be inhibited on commercially available Ti-6Al-7Nb alloy surfaces [11], which could be a problem in vivo in the presence of bacteria. There are reports in the literature on the effect of the titanium alloy, Ti-6Al-4V on staphylococci [12, 13], but not Ti-6Al-7Nb. These studies found extensive *S. aureus* and *S. epidermidis* adhesion and biofilm formation on Ti-6Al-4V compared to stainless steel, whilst Gracia et al. [14] found no significant differences. Despite these reports, little is known about the reaction of bacteria to the Ti-6Al-7Nb surface.

Intramedullary nails are often used to treat fractures of the femoral, tibial, and humeral diaphysis, and are either made from stainless steel or the titanium alloys, Ti-6Al-7Nb and Ti-6Al-4V. Standard titanium is not used for intramedullary nails because it lacks the necessary mechanical strength [15]. The use of intramedullary nails with open fractures remains controversial due to the risk of infection, and success is dependent on the skill of the surgeon, and severity of the fracture [16, 17]. Court-Brown et al. [17] reported infection rates of 1.8% in Gustilo type I open fractures, 3.8% in type II, and 9.5% in type III fractures (5.5% in type IIIa, 12.5% in type IIIb. The incidence of infection also increases if the nail is replacing an external fixation device, which had infected pins [18]. Intramedullary nail infections are often associated with non-union of the fractured bone and osteomyelitis [17]. Such infections are difficult to treat with antibiotics because the bacteria causing the infection, such as *S. aureus* and *S. epidermidis*, form biofilms on the implant surface, and often the only treatment is to remove the infected nail. Hence with the rise in bacterial resistance to antibiotics [19, 20], the need for a surface that reduces or inhibits bacterial adhesion and colonization is great.

There are reports of greater biofilm production by *S. aureus* and *S. epidermidis* on Ti-6Al-4V nails than on stainless steel nails [12, 13]. Stainless steel implants have a smooth electropolished finish [21], whilst standard titanium alloy implant surfaces are deliberately roughened for improved osseointegration [22]. Therefore, one approach to modifying the titanium alloy surface would be to polish the surface, so that the surface topography is comparable to stainless steel [11, 23]. Polished titanium alloys (Ti-6Al-7Nb) have been shown to be more cytocompatible to fibroblasts than the standard Ti-6Al-7Nb surface [11], whilst a roughened surface has also been shown to enhance bacterial adhesion [24–26]. Hence, this study describes the visualisation and quantification of *S. aureus* adhering to standard and electropolished titanium and Ti-6Al-7Nb, and standard electropolished stainless steel.

Material and methods

Materials and substrates

The standard titanium (TS) and titanium alloy, Ti-6Al-7Nb (NS) samples were made out of commercially available titanium (ISO5832/2) and Ti-6Al-7Nb (ISO 5832/11), respectively from Mathys Medical AG (Bettlach, Switzerland). Firstly the material was cut from either sheet (TS) or bar (NS), deburred, tumbled with ceramics and cleaned. The TS and NS samples were then gold anodised, whilst the electropolished surfaces TE and NE, were produced by immersing the samples in a liquid (electrolyte) and applying an electric current (Steiger SA, Switzerland), before being gold anodised. The stainless steel (SS) surfaces were made from SS (ISO 5832/1) also from Mathys Medical AG (Bettlach, Switzerland). They were cut from sheet, deburred, cleaned then electropolished as described above (Mathys Medical AG, Bettlach, Switzerland). All samples were sterilised by gamma radiation.

Surface characterisation

The surface topography of all surfaces were quantitatively measured with a non-contact ''white-light'' FRT MicroProf 200 Profilometer (Fries Research & Technology, Germany).

Roughness average (R_a—arithmetic mean of the absolute values of all points of the profile) was measured from a 2×2 mm analysis area scan at a point density of 500 points/mm. Prior to roughness calculations, a linear regression to eliminate surface inclinations was performed on each profile. Six separate points were scanned split between two samples of each surface. The surface topographies were also imaged with a Hitachi S-4700 field emission scanning electron microscope (SEM), using the lower secondary electron (SE) detector with a –50 V bias to minimise SE detection and maximise backscattered electron (BSE) detection, at an acceleration voltage of 5 kV and emission current of 40 μA.

Surface chemical analysis was carried out with X-ray photoelectron spectroscopy (XPS). Prior to surface analysis the samples were ultrasonically cleaned with Ethanol p.a. (Fluka) for 10 min to remove debris and contaminations from the packaging material. The samples were air-dried and subsequently wrapped in

aluminium foil. All spectra were recorded on a Kratos Axis Nova (Kratos Analytical, UK) using monochromated Al K_α radiation (1486.69 eV) produced at an anode power of 225 W (15 kV, 15 mA), an electron take-off angle of 90° relative to the surface plane, and an electron analyzer pass energy of 80 eV. During analysis the base pressure remained below 10^{-8} torr. For quantification, survey scans with a step width of 0.5 eV were performed on two spots of 300×700 μm^2 per sample. Data was evaluated with CasaXPS 2.3.10 (CasaXPS Ltd, UK) using relative sensitivity factors supplied with the instrument.

Bacteria culturing and visualisation

Staphylococcus aureus 8325-4 was grown in brain heart infusion broth (BHI) to an OD_{600} of around 1 at 37 °C in a shaking water bath, and used to inoculate 1 mL prewarmed BHI in four well plates containing the different surfaces, to a starting of OD_{600} of 0.05. Samples were then incubated stationary at 37 °C for 1 h. To visualise *S. aureus* adherence to the surfaces with a scanning electron microscope (SEM), adherent bacteria were fixed with 2.5% glutaraldehyde in 0.1 M PIPES (pH 7.4) for 5 min, post-fixed with 1% osmium tetroxide (OsO_4; Simec Trade AG, Zofingen, Switzerland) in 0.1 M PIPES (pH 6.8) for 1 h, dehydrated through an ethanol series (50%, 70%, 96%, 100%, 5 min each step), critical point dried in a POLARON E300 (Agar Scientific, Stansted, UK), coated with 10 nm of gold/palladium (80:20) using a Baltec MED 020 unit (Bal-Tec, Balzers, Liechtenstein), and visualised with an SEM using same conditions as described above. To quantify the density of *S. aureus* adhering, bacteria were stained with fluorescent redox dye, 5-Cyano-2,3-ditolyl tetrazolium chloride (Polysciences, Eppelheim, Germany) for 1 h, and visualised with a Zeiss Axioplan 2 Epifluorescence microscope fitted with an Axiocam camera [24]. The density of live bacteria adhering to the surface observed in each image were counted using KS400 software, and analysed statistically using a one-way ANOVA with Tukey test. This was repeated three times. Statistical significance was accepted at $p \leq 0.05$.

Table 1 Surface roughness parameter R_a for the different surfaces

Surface	Surface code	R_a (μm)
Standard micro-rough titanium	TS	0.90 ± 0.027
Electropolished TS	TE	0.19 ± 0.030
Standard micro-rough Ti-6Al-7Nb	NS	0.77 ± 0.076
Electropolished NS	NE	0.18 ± 0.037
Standard electropolished stainless steel	SS	0.19 ± 0.022

R_a is the arithmetic mean of the absolute values of all points of the profile

Results

The profilometer roughness measurements confirmed that the TE and NE surfaces were smoother than the TS and NS surfaces, as seen in Table 1. Visualisation of the surface topography of the different surfaces confirmed that the TS and NS surfaces were rougher than the TE, NE and SS surfaces with minimal background topography (Fig. 1, surfaces seen with bacteria). Despite having similar roughness measurements, the topographies of TS and NS varied considerably. TS had characteristic rugged topography, while NS had an undulating topography punctuated with protruding particles from its microstructure (Fig. 1). XPS surface chemical analysis of the various surfaces (Table 2) showed that electropolishing the TS or NE did not add any contamination to the surface chemistry of the metal, and confirmed the

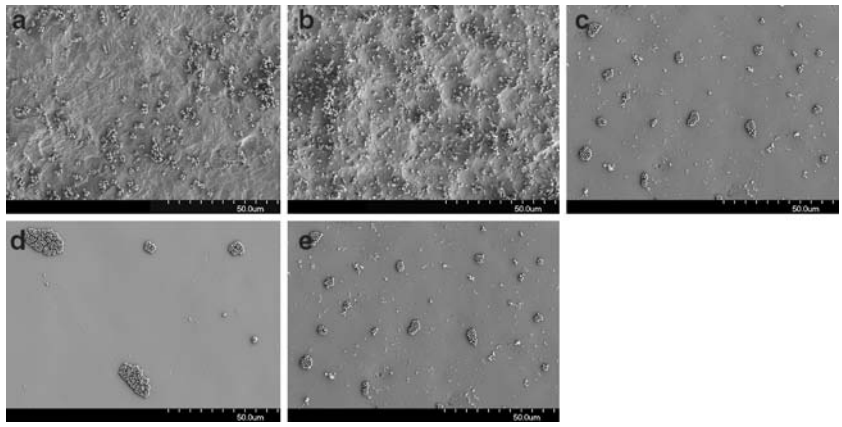

Fig. 1 SEM images of *S. aureus* adhering on (**a**) TS, (**b**) NS, (**c**) SS, (**d**) TE and (**e**) NE. Note that the bacteria on TS are scattered all over the surface in small clumps of 2–4 bacteria, whereas on NS the bacteria are in larger clumps, of more than 6 bacteria. On the electropolished surfaces (TE, NS and SS) the bacteria were found to clump in large clumps, with no small clumps of 2–4 bacteria seen

Table 2 XPS surface chemical analysis of the various surfaces

Surface	Atomic concentration [%]								
	Al 2p	C 1s	Ca 2p	N 1s	Na KLL	Nb 3d	O 1s	P 2p	Ti 2p
NS	1.7	25.5	0.0	0.95	0.75	0.2	51.15	2.1	17.55
NE	2.0	27.8	0.2	1.1	0.4	0.1	49.25	0.6	18.55
TS	n/a	26.95	0.2	0.6	1.15	n/a	50.65	3.0	17.45
TE	n/a	32.1	0.1	1.35	0.3	n/a	46.95	2.55	16.35
	Cr 2p	C 1s	Fe 2p	N 1s	Mo 3d	Na 1s	O 1s	P 2p	Ni 2p
SS	7.35	41.4	4.5	3.65	0.55	1.45	40.05	0.25	0.75

n/a—not applicable

presence of aluminium and niobium on the surfaces of NS and NE.

SEM imaging of adherent *S. aureus* on the surfaces showed that the bacteria adhered to all five surfaces, but to varying degrees (Fig. 1). Many bacteria were seen on the NS surface, but fewer were observed on the TS, TE, NE and SS surfaces. The *S. aureus* seen on the electropolished surfaces (TE, NE and SS) were in large flat clumps compared to the scattered clumps seen on the TS and NS surfaces (Fig. 1). Quantification of *S. aureus* adhesion using fluorescence microscopy found significantly more live bacteria on NS than on the other surfaces ($p \leq 0.05$), whilst there was no significant difference between the amount of bacteria on TS, TE, NE and SS surfaces (Fig. 2).

Discussion

In recent years bacteria such as *S. aureus*, have been in the news due to the increase in cases of antibiotic resistance bacteria, particularly methicillin-resistant *S. aureus* (MRSA) [19]. Due to concerns regarding the efficacy of antibiotic-loaded coatings [27–30], modifying the actual metal implant surface to inhibit or reduce initial bacterial adhesion may have potential. This in vitro study described the visualisation and quantification of *S. aureus* adhering to standard titanium, Ti-6Al-7Nb and stainless steel surfaces, and electropolished titanium and Ti-6Al-7Nb surfaces. Qualitative and quantitative results of *S. aureus* adhesion on the different surfaces correlated with each other, and showed significantly more *S. aureus* on the NS compared to NE, TS, TE and SS. The observation of more bacteria adhering to the NS surface than to SS, correlated with others who have also seen an increase in bacterial adhesion to titanium alloys compared to steel [12, 13]. There was no significant difference between TS and TE and the other two surfaces, NE and SS. Hence electropolishing Ti-6Al-7Nb (NE) decreased *S. aureus* adhesion compared to standard Ti-6Al-7Nb (NS), whilst electropolishing titanium (TE) had no significant effect on *S. aureus* adhesion.

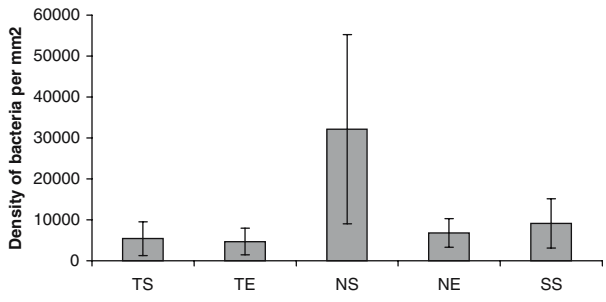

Fig. 2 Graph showing the average density of *S. aureus* adhering to the different surfaces. Note the significant difference between bacteria on NS compared to the other surfaces. The large variations observed are due to the clumping of the bacteria on the surface

Both 'standard' preparations of titanium (TS) and Ti-6Al-7Nb (NS) had similar roughness measurements, however their topographies varied. TS had characteristic rugged topography, whilst NS had an undulating topography punctuated with protruding particles from its microstructure. The standard titanium (TS) and electropolished titanium (TE) surfaces were cleaned using an acid etching method [31], which dissolved the titanium grain boundaries and removed any contamination, resulting in a micro-roughened topography (Fig. 1a), whilst the TE surfaces were electropolished prior to anodisation, resulting in the smooth finish observed with the profilometer and SEM (Fig. 1d). The Ti-6Al-7Nb surfaces (NS and NE) are composed of an α and β duplex alloy with an aluminium enriched α-phase titanium and niobium enriched β-phase titanium [15]. They were also cleaned by acid etching, however when the nitric acid and hydrofluoric acid mix were applied, the niobium enriched β-phase titanium dissolved slower than the aluminium enriched α-phase titanium, resulting in the formation of a

micro-rough surface, with niobium enriched β-phase titanium protruding particles (Fig. 1b) [31]. These protrusions are less prominent after electropolishing due to the fact that the electro-chemical etch process is non-selective [32].

The surface chemical analysis (Table 2) showed that electropolishing TS or NS did not introduce any contamination to the surface chemistry of the metal surfaces which could have possibly affected bacterial adhesion. However, during surface production an oxide layer is formed on the surfaces by anodisation. It is known that the presence of aluminium and niobium on the surface of NS affect the oxide layer composition, which in turn affects protein adsorption and conformation to the surface, and thus could influence bacterial adhesion [31, 33]. The presence of the niobium enriched β-phase titanium protrusions on the NS surface could also influence bacterial adhesion. The titanium alloy Ti-6Al-4V is also used in osteosynthesis implants, and it has been shown that *S. epidermidis* have a higher affinity to vanadium than to the titanium and aluminium on this surface. As with Nb, the vanadium in Ti-6Al-4V also enriches the β-phase of the surface [34], and Nb and V are in the same group in the periodic table, and could hence have the same influence over staphylococci adhesion as a significant decrease in adhesion was observed on NE, which has less prominent niobium enriched β-phase titanium protrusions on its surface (Fig. 1). It has also been suggested that polishing NS increases the amount of Al_2O_3 present on the surface [31], which would change the isoelectric point of the surface [31, 33], and thus influence bacterial adhesion [9, 25, 35, 36]. However the surface chemical analysis in this present study (Table 2) did not show any significant increase in Al surface composition after electropolishing.

In the case of the TS and TE the oxide layers are similar in thickness [32] and elemental composition (as seen in Table 2), so the surface charge on these two surfaces would also be comparable. Neither TS or TE had protrusions on their surfaces, thus electropolishing titanium (TE) did not significantly affect *S. aureus* adhesion compared to standard titanium (TS). Hence, despite previous publications reporting that surface charge and surface chemistry influence bacterial adhesion [24, 26, 35–38], this study would indicate that small surface micro-protrusions such as those caused by the enriched β-phase on NS affect *S. aureus* adhesion. Changes in general micro-roughness where micro-protrusions are absent, as seen from TS to TE, do not affect *S. aureus* adhesion.

Conclusions

This present study found that electropolishing Ti-6Al-7Nb surfaces (NE) significantly decreased the amount of *S. aureus* adhesion compared to the standard Ti-6Al-7Nb (NS), which had a higher affinity to the bacteria than the other surfaces tested. The study would indicate that small surface micro-protrusions or that the presence of niobium on the surface increase *S. aureus* adhesion. However, this study only looked at the adhesion of one strain of *S. aureus*, and it is known that different bacteria, including different *S. aureus* strains, adhere and colonise implants differently, and that such adhesion is dependent on whether the bacteria form a biofilm or slime layer [14, 26]. Biofilm forming strains are more adherent than non-biofilm forming strains, and it has been shown that biofilms play an important role in relation to bacterial adherence and multiplication in implant-associated infections [13]. In the future, it could be of interest to study the effect of this surface on the adhesion of other bacteria such as *S. epidermidis*, and also producing artificial, representative microtopography surfaces of other chemistries to completely prove the hypothesis that the micro-protrusions or presence of niobium increase bacterial adhesion.

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References

1. M. KHATOD, M. J. BOTTE, D. B. HOYT, R. S. MEYER, J. M. SMITH and W. H. AKESON, *J. Trauma* **55** (2003) 949
2. S. E. CRAMTON, C. GERKE, N. F. SCHNELL, W. W. NICHOLS and F. GOTZ, *Infect. Immun.* **67** (1999) 5427
3. P. FRANCOIS, P. VAUDAUX, N. NURDIN, H. J. MATHIEU, P. DESCOUTS and D. P. LEW, *Biomaterials* **17** (1996) 667
4. D. J. STICKLER and R. J. C. MCLEAN, *Cells Mater.* **5** (1995) 167
5. S. ARENS, U. SCHLEGEL, G. PRINTZEN, W. J. ZIEGLER, S. M. PERREN and M. HANSIS, *J. Bone Joint Surg. Br.* **78** (1996) 647
6. C. C. CHANG and K. MERRIT, *J. Orthop. Res.* **12** (1994) 526
7. S. PERREN *Eur. Cell Mater.* **1** (2001) 2
8. G. A. MELCHER, B. CLAUDI, U. SCHLEGEL, S. M. PERREN, G. PRINTZEN and J. MUNZINGER, *J. Bone Joint Surg. Br.* **76** (1994) 955
9. A. GRISTINA, C. HOBGOOD, L. WEBB and Q. MYRVIK, *Biomaterials* **8** (1987) 423
10. S.C. WOODWARD and T. N. SALTHOUSE, in "Handbook of Biomaterial Evaluation" (Collier Macmillan Pub, London, 1986) p. 364

11. D.O. MEREDITH, L. ESCHBACH, M. RIEHLE, A. S. G. CURTIS and R. G. RICHARDS, *J Biomed Mater Res* **75** (2005) 541
12. M. DELMI, P. VAUDAUX, D. P. LEW and H. VASEY, *J. Orthop. Res.* **12** (1994) 432
13. K. Y. HA, Y. G. CHUNG and S. J. RYOO, *Spine* **30** (2005) 38
14. E. GRACIA, A. FERNANDEZ, P. CONCHELLO, A. LACLERIGA, L. PANIAGUA, F. SERAL and B. AMORENA, *Int. Orthop.* **21** (1997) 46
15. J.A. DISEGI, in ''Titanum–6% Aluminium–7% Niobium Implant material'' (AO Technical Commission, 1993)
16. R.P. CLIFFORD, in ''AO Principles of Fracture Management'' (AO Publishing, Thieme-Verlag, Stuttgart, 2000) p. 617
17. C. M. COURT-BROWN, J. F. KEATING and M. M. MCQUEEN, *J. Bone Joint Surg. Br.* **74** (1992) 770
18. D. J. MAURER, R. L. MERKOW and R. B. GUSTILO, *J. Bone Joint Surg. Am.* **71** (1989) 835
19. EUROPEAN ANTIMICROBIAL RESISTANCE SURVEILLANCE SYSTEM ANNUAL REPORT (2002).
20. F. D. LOWY, *N. Engl. J. Med.* **339** (1998) 520
21. J.A. DISEGI, in ''Wrought 18% Chromium–14% Nickel–2.5% Molybdenum Stainless Steel Implant Material'' (AO Technical Commission, 1998).
22. D. BRUNETTE, P. TENGVALL, M. TEXTOR and P. THOMSEN, in ''Titanium in Medicine''(Springer-Verlag, Berlin Heidelberg New York, 2001)
23. R. LANGE, F. LUTHEN, U. BECK, J. RYCHLY, A. BAUMANN, and B. NEBE, *Biomol. Eng.* **19** (2002) 255
24. L. G. HARRIS, S. TOSATTI, M. WIELAND, M. TEXTOR and R. G. RICHARDS, *Biomaterials* **25** (2004) 4135
25. M. QUIRYNEN and C. M. BOLLEN, *J. Clin. Periodontol.* **22** (1995) 1
26. C. C. VERHEYEN, W. J. DHERT, J. M. BLIECK-HOGERVORST, T. J. VAN DER REIJDEN, P. L. PETIT and K. DE GROOT, *Biomaterials* **14** (1993) 383
27. M. LUCKE, G. SCHMIDMAIER, S. SADONI, B. WILDEMANN, R. SCHILLER, N. P. HAAS and M. RASCHKE, *Bone* **32** (2003) 521
28. J. S. PRICE, A. F. TENCER, D. M. ARM and G. A. BOHACH, *J. Biomed. Mater. Res.* **30** (1996) 281
29. P. VAUDAUX, P. FRANCOIS, B. BERGER-BACHI and D. P. LEW, *J. Antimicrob. Chemother.* **47** (2001) 163
30. S. M. TAMBE, L. SAMPATH and S. M. MODAK, *J. Antimicrob. Chemother.* **47** (2001) 589
31. C. SITTIG, M. TEXTOR, N. D. SPENCER, M. WIELAND and P. H. VALLOTTON, *J. Mater. Sci. Mater. Med.* **10** (1999) 35
32. J. LAUSMAA, in ''Titanium in Medicine'' (Springer-Verlag, Berlin, 2001) p. 231
33. C. SITTIG, G. HAHNER, A. MARTI, M. TEXTOR, N. D. SPENCER and R. HAUERT, *J. Mater. Sci. Mater. Med.* **10** (1999) 191
34. B. J. GABRIEL, J. GOLD, A. G. GRISTINA, B. KASEMO, J. LAUSMAA, C. HARRER and Q. N. MYRVIK, *Biomaterials* **15** (1994) 628
35. R. BOS, H. C. VAN DER MEI and H. J. BUSSCHER, *FEMS Microbiol. Rev.* **23** (1999) 179
36. J. TSIBOUKLIS, M. STONE, A. A. THORPE, P. GRAHAM, V. PETERS, R. HEERLIEN, J. R. SMITH, K. L. GREEN and T. G. NEVELL, *Biomaterials* **20** (1999) 1229
37. H.J. BUSSHER, A. W. J. VAN PELT, P. DE BOER, H. P. DE JONG and J. ARENDS, *Colloids Surf.* **9** (1984) 319
38. C. H. PEREIRA DA SILVA, G. M. VIDIGAL, M. DE UZEDA and G. DE ALMEIDA SOARES, *Implant Dent.* **14** (2005) 88