Colloids and Surfaces B: Biointerfaces 88 (2011) 648–655

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Electrochemically induced anatase inhibits bacterial colonization on Titanium Grade 2 and Ti6Al4V alloy for dental and orthopedic devices

Carmen Giordano[a], Enrica Saino[b,c], Lia Rimondini[f,*], Maria Pia Pedeferri[a], Livia Visai[b,c,d,e], Alberto Cigada[a], Roberto Chiesa[a]

[a] Dipartimento di Chimica, Materiale e Ingegneria Chimica "G. Natta", Politecnico di Milano, Milano, Italy
[b] Department of Molecular Medicine, Viale Taramelli 3/B, University of Pavia, 27100 Pavia, Italy
[c] Center for Tissue Engineering (CIT), University of Pavia, Via Ferrata 1, 27100 Pavia, Italy
[d] Fondazione S. Maugeri, IRCCS, Istituto Scientifico di Pavia, Via S. Maugeri, 4, 27100 Pavia, Italy
[e] International Centre for Studies and Research in Biomedicine (I.C.B.), 16 a, Bd. de la Foire, L-2015 Luxembourg
[f] Dipartimento di Scienze Mediche, Università del Piemonte Orientale, Via Solaroli, 17, Novara, Italy

ARTICLE INFO

Article history:
Received 8 February 2011
Received in revised form 26 July 2011
Accepted 27 July 2011
Available online 4 August 2011

Keywords:
Anodization
Titanium dioxide
Ti6Al4V alloy
Anatase
Biofilm
Cytocompatibility

ABSTRACT

Bacterial contamination of implanted devices is a common cause of their failure. The aim of the present study was to assess the capability of electrochemical procedures to: (a) promote the formation of anatase on the surface of commercially pure Grade 2 Ti and Ti Grade 5 (Ti6Al4V) alloy; (b) inhibit *in vitro* biofilm formation of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans* and *Porphyromonas gingivalis* and oral plaque *in vivo*, (c) preserve favorable response of osteoblasts and fibroblasts to materials surfaces. Ti Grade 2 and Ti Grade 5 were respectively anodized at two different voltages: 90 and 130 V for pure titanium; 100 and 120 V for Ti6Al4V alloy. Surface characterization was performed by scanning electron microscopy (SEM) equipped with EDS probe, laser profilometry and X-ray diffractometry. Bacterial adhesion characterization was performed either *in vitro* and *in vivo* in patients. Osteoblast and fibroblast response was evaluated by metabolic activity assessment. The higher voltage applied in the anodization treatment of pure titanium (130 V) and Ti6Al4V alloy (120 V) surfaces, compared to the untreated pure titanium and Ti6Al4V and to lower voltage treatments, resulted in a greater decrease in bacterial attachment and biofilm formation in both *in vitro* and *in vivo* experiments. In contrast, the high voltage treatments were found to promote osteoblasts and fibroblasts proliferation.

The observations indicated that the experimented high voltage anodization treatments may contribute to preserve the tissue integration and reduce bacteria colonization of titanium and titanium alloy for implantable applications.

1. Introduction

Implant-supported restorations offer a predictable treatment for tooth replacement [1,2]. Many factors influence the long term reliability of oral implants such as healthy and sealing peri-implant tissues and non-putative pathogen oral microflora [3]. In healthy conditions, peri-implant sulci are colonized by Gram positive aerobic facultative microorganisms. The flora around successful implants is similar to that recoverable around periodontal healthy teeth [4]. In contrast, several studies have identified similarities in the pathogenesis of periodontitis and peri-implantitis [5]. The plaque accumulation and its evolution toward periodontal pathogenic species have been claimed as factor implicated in the pathogenesis of peri-implantitis. As in natural dentition, the biofilm formation around a dental implant is characterized, first, by the prevalence of coccoides Gram positive forms [6], even though sophisticated detection including checkerboard DNA–DNA hybridization has shown that early colonization patterns differ between implant and tooth surfaces. Some authors [7] reported that immediately post-surgery, 5.9% of implants and 26.2% of teeth, and at week 12, 15% of implants and 39.1% of teeth, harboured *Staphylococcus aureus*. Comparing tooth and implant sites, significantly higher bacterial loads were found at tooth sites for 27/40 species after 30 min following implant placement. This difference increased to 35/40 species at 12 weeks post-surgically.

The depth of the crevice influences the probability of recovering pathogens. It has been observed that a probing depth of more than 4 mm is associated with the presence of *Porphyromonas gingivalis* [8], *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* [9,10].

Titanium based dental implants are widely and successfully used in implantology [11]. Titanium alloys present some

* Corresponding author. Tel.: +39 0321 660 673; fax: +39 0321 620 421.
E-mail address: lia.rimondini@med.unipmn.it (L. Rimondini).

0927-7765/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.colsurfb.2011.07.054

bio-mechanical advantages with respect to pure titanium: higher strength resistance makes them good candidates for highly stressed devices such as mini-implants for dental application and orthopedic devices.

Microbial colonization and antibacterial activity on metallic implant materials have been reported by *in vitro* and *in vivo* studies [12–16]. Titanium itself does not show antibacterial activity, but its surfaces may be conveniently modified in order to alter bacteria colonization [17–21]. Surface composition, morphology and nanostructure strongly affect bacteria interaction and also bone, gingival and skin cell response [22–24]. Several industrial finishing processes, such as metal coatings, sandblasting, chemical etching, have been commonly applied. Moreover, chemical and electrochemical modification aimed at improving titanium and titanium alloy biocompatibility are able to modify surface morphology and roughness at different scales, changing the crystalline structure of titanium oxide layer that spontaneously grows on titanium and titanium alloy devices once in contact with the air [25]. Thanks to suitable manufacturing procedures, surface features of titanium dioxide might be designed to enhance metal–tissue integration *in vivo*, by proper surface roughness, porosity and nanostructuration [26,27].

The TiO_2 at the surface may be amorphous, partially or fully crystalline. The most frequent crystal forms assumable are rutile but two rarer polymorph oxides are known: brookite that crystallizes into an orthorhombic system, and anatase. The latter, being rutile, belongs to the tetragonal system but differs from it in the longer *C*-axis. This configuration is responsible for some physical characteristics of anatase such as super-hydrophilicity or semiconductor properties which promote chemical catalysis [28]. The photocatalytic properties of TiO_2 are well known in material sciences especially in environmental and antipollution applications [29]. Since a long time ago TiO_2 crystallized in the form of anatase was demonstrated to have a potential application in medicine because of the antimicrobial effect observed in both *in vitro* and *in vivo* experiments [25,30–33]. Moreover, it has been reported that the antibacterial effects of anatase are enhanced by UV light illumination [34].

The aims of the present paper were to assess: (1) the capability of electrochemical procedures to promote the formation of anatase TiO_2 superficial layer on Ti Grade 2 and Ti Grade 5; (2) the effect of resulting surfaces with regards the *in vitro* cytocompatibility of fibroblasts and osteoblasts; and (3) the inhibition of bacterial attachment, growth and biofilm formation on the developed materials using bacterial strains frequently involved in oral pathologies and failure of implanted devices.

2. Materials and methods

2.1. Preparation of materials

Ti Grade 2 (pure titanium) and Ti Grade 5 (Ti6Al4V alloy) squared sheet specimens (10 mm × 10 mm × 1 mm) were obtained respectively from a Grade 2 commercially pure titanium and a Ti6Al4V alloy sheet (Toresin Titanium, Padova) using a mechanical cutter. The obtained specimens were cleaned with acetone and an ultrasonic bath. Electrochemical treatments were performed in 0.5 M of sulphuric acid solution at room temperature. A Titanium Grade 2 sheet (50 cm × 15 cm) was properly circularly bended inside a beaker to surround the anode specimens and to form the cathode and the electrochemical circuit. Current was maintained at 0.04 A, corresponding to a density of 0.02 A/cm^2; the voltage was increased until the final selected value was reached (90 and 130 V for Titanium Grade 2; 100 and 120 V for Titanium Grade 5). The electrochemical treatment samples were then washed in an ultrasonic bath with distilled water, air dried, packaged in blisters and sterilized with gamma radiation at 25 kGy. The following list shows the characteristics of the materials, the electrochemical condition used for surface modification and the codes used to identify samples.

- Ti Grade 2; not treated (code: A)
- Ti Grade 2: anodized at V = 90 V (code: B)
- Ti Grade 2: anodized at V = 130 V (code: C)
- Ti Grade 5 (Ti6Al4V alloy): not treated (code: D)
- Ti Grade 5 (Ti6Al4V alloy): anodized at V = 100 V (code: E)
- Ti Grade 5 (Ti6Al4V alloy): anodized at V = 120 V (code: F)

2.2. Morphological and microstructural characterization of the material surfaces

Specimen surface morphology and qualitative chemical composition were assessed by scanning electron microscopy (SEM) (StereoScan 360, Cambridge, Cambridge, UK) equipped with an EDS without any coating. Microstructural characterization was performed by thin film X-ray diffractometry (XRD) (Philips PW 1710, Philips Electronic Instruments, Inc., Mahwah, NJ) to assess the presence of anatase crystalline titanium oxide. Cu Kα radiation generated at 40 kV and 40 mA was used. The instrument was configured with a 1° divergence and 0.2 mm receiving slits. The samples were prepared using the front loading of standard aluminium sample holders which are 1 mm deep, 20 mm high and 15 mm wide. Roughness parameters were calculated on a single measurement performed on a 1.25 mm × 1.75 mm area acquired using a (NC-LPM) 3D laser profilometer (UBM-Microfocus Compact, NanoFocus AG, Germany). The following 3D roughness parameters, calculated according to ISO 25178, were considered:

- Sa: the arithmetic mean height of the surface;
- Sz: maximum height of the surface;
- Ssk: the symmetry of the distribution of the profile height on an ideal average surface (skewness of height distribution).

Contact angle measurements were performed using an optical instrument (Dataphysics Instruments, Mod. OCA 15 Plus) equipped with a specific software (32-bit software SCA20) capable to control the liquid drop deposition rate and volume on the samples surface. The liquid used for the test was distilled water, the drop volume was 2 μl and the flow rate was set at 1 μl/s. Four to five measurements were performed for each sample. After the deposition of each drop, the contact angle was measured through image processing. All the measurements were performed at room temperature.

2.3. In vitro cells assays

2.3.1. Osteoblasts and fibroblasts response assessment

Each material was prepared as disks 1.1 cm wide. All experiments were done in triplicate for each time point. The experiments were repeated 4 times.

Osteoblast cell line (MG63-human osteosarcoma from ECACC 86051601) and murine fibroblast cell line (L929 from ECACC 85011425) were cultured and seeded onto each material placed in a 24-well tissue culture plate and seeded with 50 μl of the appropriate cell density suspension. Cells were allowed to adhere for 1 h then flooded with 1 ml of culture medium and placed back into the incubator. A cell density of 2×10^5 cells/ml on each specimens was used for both metabolic and the proliferation study for SEM morphology and adhesion assessment as described elsewhere [21,34]. Tissue culture plastic polystyrene (Corning-Costar, Italy) was used as control.

For morphology and adhesion assessment, cells were seeded at a density of 2×10^5 cells/specimen. 24 and 48 h post seeding, samples were treated with glutaraldehyde 2% solution in Na cacodylate

buffer 1 M for 4 h, rinsed in buffer, followed by an increasing concentration of ethanol solutions (70, 80, 90, 100 for 10 min each), and finally air dried, as described elsewhere [34]. Lastly, after sputter coating (Sputter coater Edwards S150B) samples were observed by SEM (Cambridge Instruments, Stereoscan 360).

For metabolic activity evaluation, cells were seeded (2×10^5 cells/sample) and analyzed at the selected time points (1, 3, 10 days), as previously described [34]. Briefly, at the selected time points, culture medium was replaced with Almar Blue™ solution (Serotec, Prodotti Gianni S.p.A., Italy) and, after 4 h of incubation, the supernatants were transferred into a novel plate and evaluated at 570 nm using a plate reader (Tecan Genius Plus, Tecan, Belgium). The samples, flooded with fresh medium, were then placed back in the incubator until the selected time points.

2.4. In vitro and in vivo bacterial assays

2.4.1. Bacterial strains and growth conditions

Streptococcus mutans (CCUG 35176) and *P. gingivalis* (CCUG 25211) were purchased from the Culture Collection of the University of Göteborg (CCUG); *S. aureus* (NTCC 8325-4) and *Staphylococcus epidermidis* RP62A (ATCC 35984) were acquired from the National Type Culture Collection (NTCC). All strains were cultured in Brain Heart Infusion (BHI, Difco, CA); to improve *S. mutans* and *P. gingivalis* growth, 10% (v/v) inactivated horse blood serum (Oxoid, Italy) was added to the medium as a supplement. The cultures of *S. mutans*, *S. aureus* and *S. epidermidis* were statically incubated for 16 h at 37 °C under aerobic conditions; *P. gingivalis* culture was statically grown for 16 h at 37 °C under strictly anaerobic conditions (Anaerogen Compact TM, Oxoid, Italy). These cultures, used as a source for the experiments, were reduced to a final density of 1×10^{10} cells/ml as determined by comparing the OD_{600} of the sample with a standard curve relating OD_{600} to cell number.

2.4.2. Bacterial adhesion assay

An overnight culture of bacterial cells (1×10^4 cells/sample) were seeded onto each sample test and incubated in static condition at 37 °C for 3 h. Loosely adhering bacteria were removed by gently washing the specimens with phosphate buffer solution (PBS) and the total viable count (TVC) estimation was performed as previously described [21–25]. Briefly, the samples with the adherent bacterial cells were dispersed into 1 mL sterile Ringer solution (Oxoid, Italy) by vortex for 5 min. At the end of the incubation time, serial dilutions of the bacterial cell suspensions were prepared and 0.1 ml of each dilution was deposited onto BHI agar (Bacto agar, Difco, CA) plates. The plates were incubated for 24–48 h at 37 °C (in strictly anaerobic conditions for *P. gingivalis*, or in a CO_2 incubator for *S. mutans* to improve colony growth) and the number of colonies counted.

2.4.3. Bacterial proliferation assay

Cells at the concentration of 1×10^2/specimen were seeded onto each material and incubated at 37 °C for 24 h. At the end of the incubation time, an aliquot of the bacterial suspension was serially diluted, deposited on BHI agar plate an incubated as previously indicated.

At the end of the bacterial adhesion and proliferation experiment, the mean TVC values were calculated for each sample and the results were expressed as colony forming units (CFU)/cm^2.

The experiments were done in triplicate and repeated 4 times.

2.4.4. Biofilm growth

For biofilm growth, overnight cultures of bacterial strains, *in vitro* biofilm-formers, were diluted at 1:200 in Tryptone Soy Broth (TSB) or BHI containing 0.25% glucose (*S. aureus* and *S. epidermidis*), 1% glucose (*S. mutans*), or no glucose (*P. gingivalis*) respectively and incubated under the conditions reported above [40,41]. Aliquots (400 μl) of the diluted bacterial suspensions were inoculated directly onto the different type of titanium samples deposited at the bottom of the 24 wells and incubated for 24 h at 37 °C. Biofilm formation by bacteria was detected by the method described in the literature [37]. Briefly, biofilms formed on the plates were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, pH 7.4) to remove the planktonic cells. Then, the cells were fixed with 95% ethanol for 10 min and stained with 0.1% crystal violet for 15 min, and after several washings, the wells were air dried. For a quantitative estimation of biofilms, crystal violet was solubilized with 10% glacial acetic acid and absorbance of the solubilized dye was determined at 595 nm in a microplate reader (Microplate Reader 680; BioRad Laboratoires, Inc., Hercules, CA).

The experiments with biomaterials were done in triplicate and repeated 4 times.

2.4.5. In vivo biofilm formation

In vivo evaluation protocol obtained the *nulla osta* by the Local Ethical Committee for Clinical Trials.

Eight volunteers (3 males and 5 females), aged between 20 and 25 years, with good oral and general health conditions and high standards of oral hygiene (Full Mouth Plaque Score less than 20%) were recruited. None of them had used antibiotics or antibacterial solution during the three months before the experiment. All the participants started to use the same toothpaste without fluoride (Biorepair–Coswell), 2 weeks after the experiment.

Full arch impressions were taken from each subject in order to make gypsum casts and then silicone appliances. After occlusal adaptation of the appliances, titanium and alloy disks, 5 mm wide, were thermically fixed on their buccal-lateral sides. Six disks, two for each sample, were installed, in random order from distal to mesial parts of each appliance.

Then, the volunteers wore the appliances consecutively for 24 h, removing them only for meals and without performing any oral hygiene procedure.

After 24 h that the specimens were removed from the appliances and processed for SEM. A total area of 6300 μm^2 composed of four randomly selected fields of 35 μm × 45 μm, was examined for each specimen. The density of bacteria observed on each specimen was measured using an index as the sum of the different scores observed on the four fields.

The scores considered were the following: 0 = no bacteria observed; 1 = number of bacteria ranging between $>0 \leq 10$; 2 = number of bacteria ranging between $>10 \leq 100$; 3 = number of bacteria > 100.

2.5. Statistical analysis

Statistical analysis was performed with ANOVA test for variables regarding profile characterization and cell culture data. The nonparametric Friedmans's ANOVA and Wilcoxon's exact test with Monte Carlo method, and Kruskal–Wallis ANOVA test followed by post-hoc Dunn's test was used for probability calculation for multiple comparisons using data from *in vitro* and *in vivo* microbiological experiments. Two-tailed probability was considered. Threshold for significance was set for $p < 0.05$.

3. Results

3.1. Morphological and microstructural characterization of the material surfaces

Electrochemical treatment did not lead to any significant modification of the surface morphology at a microscale in both Ti Grade 2

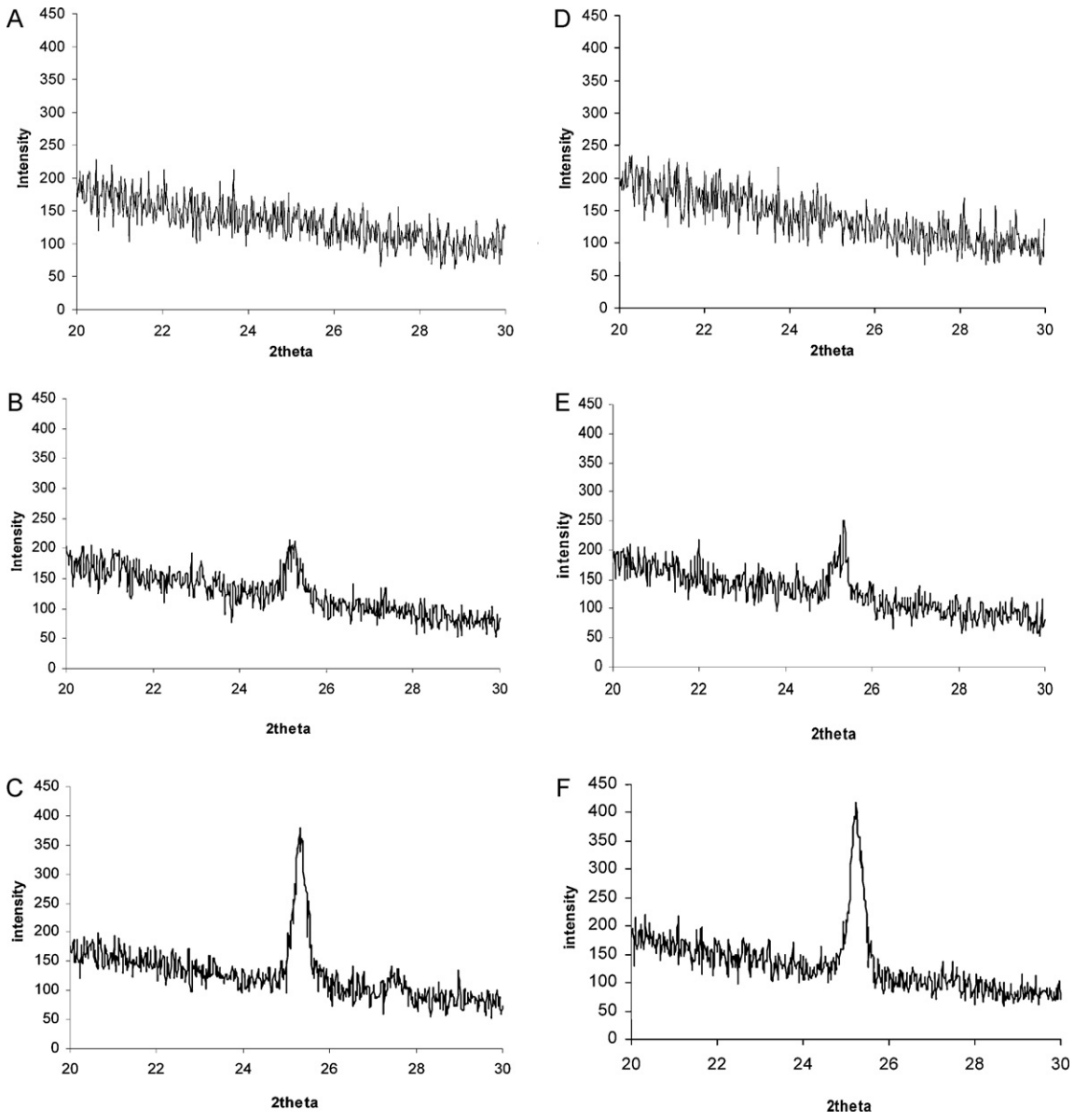

Fig. 1. Thin film X-ray diffractometry (XRD) of anodized samples. Microstructural characterization was performed by (XRD) to observe the presence of anatase (peak at 25°) on sample surfaces. Samples: Ti Grade 2: not treated (A); Ti Grade 2: anodized at V = 90 V (B); Ti Grade 2: anodized at V = 130 V (C); Ti6Al4V: not treated (D); Ti6Al4V: anodized at V = 100 V (E); Ti6Al4V: anodized at V = 120 V (F). A small rutile peak is observable in sample F.

and Ti Grade 5 specimens. Qualitative X-ray diffractometric analysis showed, for both anodized commercially pure titanium (Fig. 1B and C samples) and Ti6Al4V (Fig. 1E and F samples) sample surface, the presence of anatase as indicated by the intensity of the peaks, in the 2θ range 20–30°. The peak was not present for the not treated samples (Fig. 1A and D samples). Anatase relative amount, as indicated by the intensity of the peaks close to 25°, was lower for the samples anodized with a lower voltage (B and E samples) compared to the substrata anodized at higher voltage (C and F samples) regardless of the base material composition. No anatase peak was observed on not treated Ti Grade 2 and Ti Grade 5 specimens A and D.

Roughness results are summarized in Table 1. The high voltage anodized samples showed a slight higher roughness Sa and Sz for both Ti Grade 2 and Ti Grade 5 materials. Ssk parameter did not show any important differences in the peak and valley distributions for all materials.

Contact angle measurements analysis results, shown in Fig. 2, provided similar values for all tested material with one exception: a slight decrease of the contact angle for material B (i.e., Ti Grade 2 anodized at 90 V). Apart of this exception, no specific trends could be observed.

3.2. Osteoblast and fibroblast in vitro cell response

MG63 osteosarcoma and L929 fibroblastic cell morphology and adhesion assessment at 24 and 48 h post seeding showed healthy, well-spread cells on both not treated and anodized substrata (data not shown).

Table 1
Roughness parameters.

	A	B	C	D	E	F
Sa	0.306	0.309	0.355	0.342	0.436	0.506
Sz	2.510	2.414	2.700	2.628	2.941	3.858
Ssk	−0.131	−0.335	−0.101	−0.235	0.006	0.005

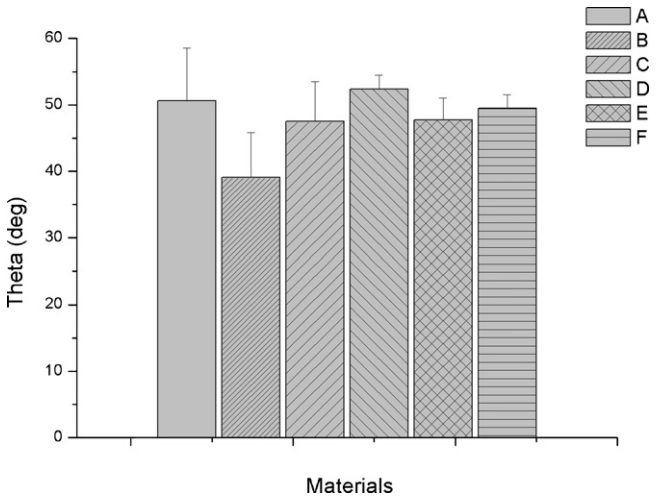

Fig. 2. Contact angle measurement for the tested materials.

The metabolic activity assay shows comparable values for all the materials ($p > 0.05$) after 1 day of incubation and a remarkable increasing trend from day 1 to day 10 for all the materials and both cell lines used (Fig. 3a and b) (Friedman's ANOVA, and Wilcoxon's test $p < 0.01$). In particular, MG-63 human derived osteosarcoma cells recorded higher values of optical densities when cultured on anodized surfaces compared to untreated ones for both titanium and titanium alloy substrata in the latest stages of experiment (Kruskal–Wallis ANOVA and Dunn's test, $p < 0.05$).

3.3. Bacterial adhesion and proliferation

In vitro bacterial adhesion and growth inhibition on untreated Grade 2 or Grade 5 Ti and their electrochemical modified surfaces with *S. mutans*, *P. gingivalis*, *S. aureus* and *S. epidermidis* strains were assessed (Figs. 4 and 5).

As shown in Fig. 4a, after 3 h incubation the number of CFUs of the indicated bacterial strains was significantly lower on the 130 (C) and 90 (B) V anodized Ti Grade 2 surfaces compared to the untreated Ti Grade 2 (A) ($p < 0.05$). Similarly, after 24 h incubation the number of CFUs/cm^2 detected was significantly lower on the B and C samples compared to the A samples ($p < 0.05$) (Fig. 4b).

Specimens belonging to B and C samples harboured different amount of *S. aureus* and *S. epidermidis* after 3 h ($p < 0.05$) but not *S. mutans* and *P. gingivalis cells* ($p > 0.05$).

As shown in Fig. 5a, after 3 h incubation the numbers of bacteria belonging to all strains and attached onto the materials strains were less onto the 120 (F) and 100 (E) V anodized Ti Grade 5 surfaces compared with the untreated Ti Grade 5 (D) ($p < 0.05$). In particular, F specimens harboured less *S. aureus* than E ones after 3 h and less *S. epidermidis* and *P. gingivalis* after 24 h ($p < 0.05$) (Fig. 5b)

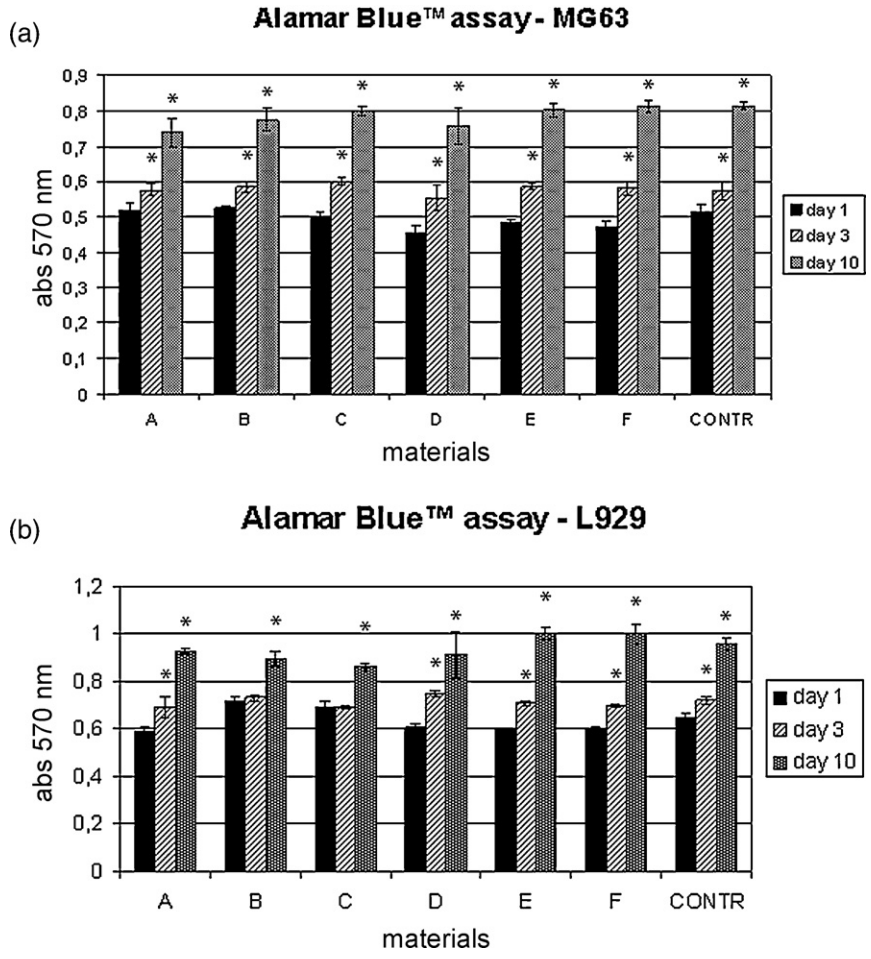

Fig. 3. Assessment of osteoblasts-like MG63 and fibroblasts L929 metabolic activity on the different substrata. Cells cultured for 1, 3, 10 days and Alamar Blue™ assay. Means and standard deviations. Samples: Ti Grade 2 not treated (A); Ti Grade 2: anodized at V = 90 V (B); Ti Grade 2: anodized at V = 130 V (C); Ti6Al4V: not treated (D); Ti6Al4V: anodized at V = 100 V (E); Ti6Al4V: anodized at V = 120 V (F). *Friedman's ANOVA, and Wilcoxon's test $p < 0.01$.

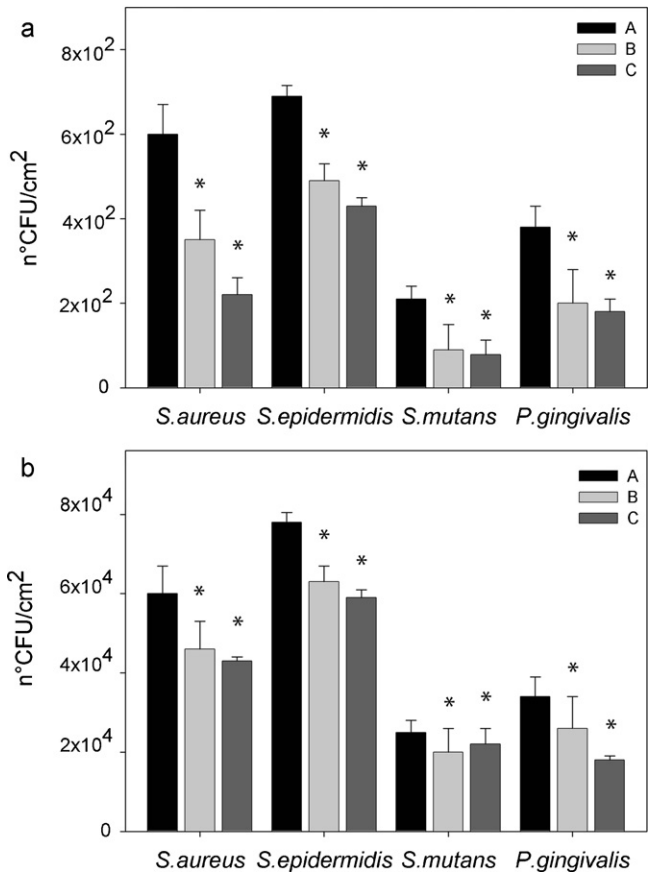

Fig. 4. Total viable count of *S. aureus* and *S. epidermidis*, *S. mutans* and *P. gingivalis* cells on Ti Grade 2 after (a) 3 h, and (b) 24 h. Means and standard deviations. Kruskal–Wallis ANOVA and post-hoc Dunn's tests to compare untreated vs. treated samples. $^{*}p < 0.05$.

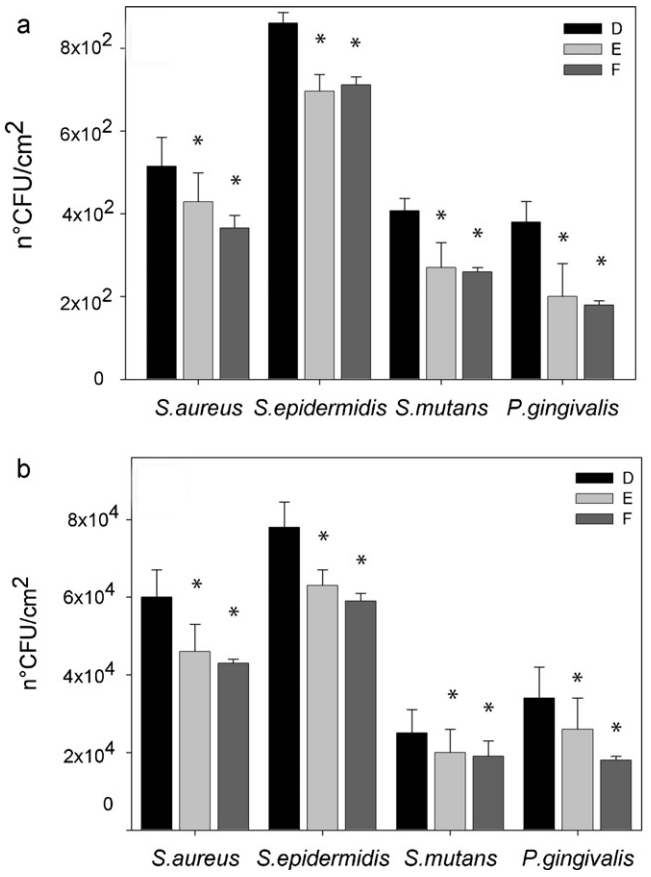

Fig. 5. Total viable count of *S. aureus* and *S. epidermidis*, *S. mutans* and *P. gingivalis* cells on Ti Grade 5 specimens after (a) 3 h, and (b) 24 h. Means and standard deviations. Kruskal–Wallis ANOVA and post-hoc Dunn's tests to compare untreated vs. treated samples. $^{*}p < 0.05$.

3.4. In vitro biofilm growth

A critical hallmark of infections on biomaterials is the ability of the bacteria to grow as a biofilm, a sessile community of cells that is embedded in an extracellular polymeric matrix. To investigate this matter, we tested the *in vitro* ability of the chosen bacterial strains to produce slime and we considered its formation as parameter to evaluate the efficacy of the electrochemical treatments applied onto titanium surfaces in reducing the biofilm formation.

As shown in Fig. 6, the biofilm growth for the indicated bacterial strains was lower on the Ti Grade 2 surfaces anodized at 130 V (C) (Fig. 6a) and on the Ti Grade 5 surfaces anodized at 120 V (F) (Fig. 6b) compared to the untreated Ti surfaces A (Ti Grade 2) and D (Ti Grade 5), respectively ($p < 0.05$). No significant differences were observed between the titanium surfaces treated at higher and lower voltages either on Ti Grade 2 or Grade 5 surfaces. The reduction was particularly evident for *S. mutans* and *P. gingivalis* on Ti Grade 2 surfaces C and for *S. aureus* and *S. epidermidis* on Ti alloy surfaces F.

3.5. In vivo experiments

The amount of microbial cells early adhering to the substrates varied significantly among the different materials ($p < 0.001$) (Fig. 7). The electrochemical treatments had a detrimental effect on the surfaces of Ti Grade 2, increasing ($p < 0.05$) the amount of adhering cells on B and C samples in comparison with those not treated (A), which appeared, at SEM observation, colonized by few and isolated groups of cocci. The biofilm grown on the electrochemically modified Ti Grade 2 appeared widespread and characterized by the co-aggregation of cocci rods and filamentous bacteria (SEM data not shown).

In contrast, electrochemical treatment on Ti Grade 5 specimens seems to have had differing effects on their exposure to biofilm colonization ($p < 0.05$). The treatment with the highest voltage (F) provoked a worsening of the contamination ($p < 0.05$) (Fig. 7) characterized by a thicker and composite biofilm formation, at SEM observation, in comparison with not treated surfaces (D), while the electrochemical treatment with 100 V did not determine any significant increase in bacterial contamination when compared with both not treated Ti Grade 5 and not treated Ti Grade 2.

4. Discussion

One of the main strategies to improve the success rate of titanium oral implants or to reduce the side-effects of trans-dermal orthopedic devices is to develop surfaces less prone to biofilm colonization. This goal may be achieved by many different technologies based on varying approaches including modification of the microtexture, grafting of peptides and organic microbial agents onto the surface or doping the surface of the devices with metallic ions with demonstrated antibacterial activity such as Zn or Ag [19,20,38–42].

The aim of the present paper was to explore the possibility to obtain an antifouling surface by increasing the anatase content of the superficial layers of Ti Grade 2 and Ti Grade 5 (Ti6Al4V alloy) and by means of anodic electrochemical procedures. The experimental sets included both *in vitro* and *in vivo* experimentation and aimed to assess the biocompatibility of the materials obtained and the

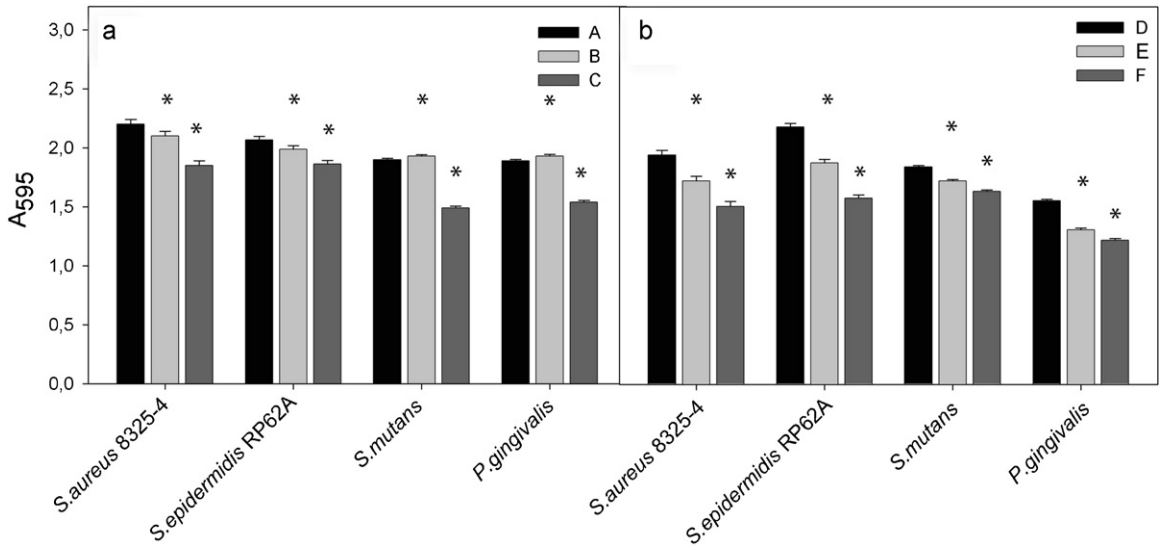

Fig. 6. Biofilm formation for the indicated bacterial strains on Ti Grade 2 and 5. Crystal violet assay for the evaluation of the slime produced by the strains. Means and standard errors. Kruskal–Wallis ANOVA and post-hoc Dunn's tests to compare untreated vs. treated samples. $^{*}p < 0.05$.

effectiveness toward putative pathogens that may be recovered on intra-oral or trans-dermal devices.

The anodic electrochemical procedures described and assessed in our study were able to promote anatase formation on both Ti Grade 2 and Ti Grade 5 surfaces. The amount of anatase was related to the electrochemical condition used. In particular anatase increased in Ti samples anodized at the higher voltage. The same effect was noted for Ti Grade 5 specimens. This is related to the increase in thickness of the oxide layer during the anodization treatment: higher voltages correspond to thicker oxide layers.

Surface roughness is known to play a major role in both osseointegration quality and rate of titanium dental implants [43]. Systematic review have been recently pointed out that moderately rough surfaces ($1\,\mu m < Sa \leq 2\,\mu m$) seem to improve bone responses more than those smooth ($0.5\,\mu m < Sa \leq 1\,\mu m$) or highly rough ($Sa > 2\,\mu m$). However, no conclusive data may be provided because the studies reviewed were carried out in different animals models and topography were evaluated using different technologies [44].

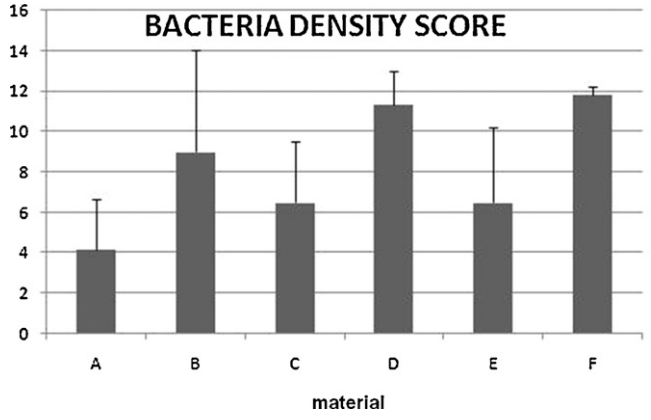

Fig. 7. Bacteria density score in the biofilm observed in *in vivo* early colonization experiments. Data represent the means and standard deviations. Comparison among the groups, Kruskal–Wallis test ($p < 0.001$). Comparison among Ti Grade 2 samples (A–C), Kruskal–Wallis test ($p < 0.01$); post-hoc Dunn's test A vs. B ($p < 0.05$); A vs. C ($p < 0.05$); B vs. C not significant. Comparison among Ti Grade 5 samples (D–F), Kruskal–Wallis test ($p < 0.05$); post-hoc Dunn's tests D vs. E not significant; D vs. F ($p < 0.05$); D vs. F ($p < 0.05$).

In contrast, topographies in the nanometer range have been demonstrated to affect the initial stage of healing and it is specially addressed to promote proteins adsorption, osteoblastic cells adhesion and the rate of bone tissue healing in the peri-implant region.

The anodization treatments performed and analyzed in this study were found not capable to significantly modify the surface roughness of pure titanium or Ti6Al4V measured by non-contact laser profilometry. We may state that the biological results obtained in this study cannot be related to any surface roughness difference among the studied materials, at least in the micrometric scale on which the NC-LPM roughness measurements were performed.

When anodized surfaces were compared for cell–materials interaction, it was noticed that anodized surfaces showed different surface properties, which led to variations in cell–materials interactions [45]. Experimentally, changes in these surface properties on titanium through anodization influence the concentration and conformation of adsorbed proteins to alter cellular interactions. Another important factor in the adhesion and activation of cells is the wettability of the material surface. The possibility to produce hydrophilic surface for titanium implants is highly desirable even if it is not easy to obtain merely altering the surface chemical composition by anodization process. The anodization treatments performed on Ti Grade 2 and Ti grade 5 did not significantly modify the wettability: only a slight decrease of contact angle values could be observed on material B (Ti Grade 2 anodized at 90 V), but no specific and experimentally significant tendencies were detected.

Many factors affect the initial attachment of microorganisms to inert substrata, and their subsequent retention or removal/detachment, including the physical and chemical nature and location of the substratum, the type of organic material and microorganisms potentially fouling the surface, and the nature of the interface [46].

The experimental results of our viability study performed with *S. aureus* 8325-4, *S. epidermidis* RP62A, *S. mutans*, and *P. gingivalis* strains showed antibacterial activity of the anodized surfaces containing anatase with respect to the untreated Ti Grade 2 and Ti Grade 5 surfaces. These results are in accordance with other recent studies which have shown the bactericidal effects of titanium dioxide nanoparticles [47] on the viability of different microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, *S. aureus*,

Enterococcus hirae, and *Bacteroides fragilis* and also methicillin-resistant *S. aureus* (MRSA) suspensions. These observations were confirmed in both *in vitro* assay and in an *in vivo* animal model by inserting pins into the femurs of rabbits [48].

The results of our study on bacterial biofilm formation are quite interesting: biofilm was significantly reduced on the anodized Ti Grade 2 and Ti Grade 5 surfaces in both *in vitro* and *in vivo* experiments even if, in this latter case, with different evidence. No clear reasons could explain the differences between *in vitro* and *in vivo* results observed regarding bacterial growth. Indeed the experimental conditions are quite different. In fact, it is important to highlight that *in vitro* results are obtained in a controlled way using specific bacterial strains. On the contrary the *in vivo* evaluation are affected by many varied conditions such as the presence and composition of salivary pellicle, the natural immunity factors, the removal action of mechanical forces due to mucosa movements and salivary flow, the fluctuant temperature, and the heterogeneity of the nutrients and metabolites and species involved in the biofilm formation.

As is well known, implant-related infection is closely related to the biofilm. The biofilm is produced by adhesion, colonization and the formation of multiple layers of bacteria, glycoprotein, and glycocalyx secreted from bacteria [49]. Once the biofilm has formed, it can be very difficult to deal with, because it is resistant to antibiotics and the host's defence mechanism [50]. Therefore, the anodization treatment here presented could be used as a prophylactic approach to reduce bacterial colonization of implanted medical devices. This conclusion is in accordance with that recently observed [51], in middle terms, in patients: the reduction of biofilm contamination of subcrestal healing screw surface of oral implants coated with anatase in comparison with those without.

It is known that anatase may play a positive role in the osteointegration process *in vivo*: thanks to its photocatalytic properties, anatase plays a key role in the precipitation of calcium phosphate *in vitro* when in contact with *simulated body fluid*, resulting in a more reliable osteointegration already observed *in vivo* [52]. In accordance with these findings, in the present study *in vitro* osteoblast and fibroblast cell response assessment showed healthy spread of MG63 and L929 cells on not treated and on anodized titanium and titanium alloy materials at both the time points selected, suggesting that the treated samples represent a suitable surface for cell colonization comparable with not treated substrata. Metabolic activity study indicated that the anodized substrata represent a suitable environment for cellular metabolism both for MG63 osteosarcoma and L929 fibroblastic cell lines, with no remarkable differences among materials and control at almost all time points selected.

5. Conclusions

Considering all the data collected, our results indicate the effectiveness of the electrochemical surface modification performed both on Ti Grade 2 and Ti grade 5 in the reduction of bacterial colonization, at the same time preserving the well known excellent biological performance of titanium, in particular when in contact with osteoblastic-like cells.

Acknowledgments

The study was partially supported by the International Centre For Studies And Research In Biomedicine (ICB) in Luxembourg, by "Project SAL-45" (Regione Lombardia, 2010), by ALMA MATER TICINENSIS Foundation (2010) for L.V., and Ricerca Sanitaria Finalizzata Piemonte 2008 for L.R.

References

[1] C.T. Ong, S. Ivanovski, I.G. Needleman, M. Retzepi, D.R. Moles, M.S. Tonetti, N. Donos, J. Clin. Periodontol. 35 (2008) 438–462.
[2] C.E. Misch, M.L. Perel, H.L. Wang, G. Sammartino, P. Galindo-Moreno, P. Trisi, M. Steigmann, A. Rebaudi, A. Palti, M.A. Pikos, D. Schwartz-Arad, J. Choukroun, J.L. Gutierrez-Perez, G. Marenzi, D.K. Valavanis, Implant. Dent. 17 (2008) 5–15.
[3] K. Subramani, R.E. Jung, A. Molenberg, C.H. Hammerle, Int. J. Oral Maxillofac. Implants 24 (2009) 616–626.
[4] A. Mombelli, Periodontology 2000 28 (2002) 177–189.
[5] J. Lindhe, J. Meyle, J. Clin. Periodontol. 35 (2008) 282–285.
[6] M. Quirynen, M. De Soete, D. van Steenberghe, Clin. Oral Implants Res. 13 (2001) 1–19.
[7] M.M. Fürst, G.E. Salvi, N.P. Lang, G.R. Persson, Clin. Oral Implants Res. 18 (2007) 501–508.
[8] A. Rutar, N.P. Lang, Buser D.F D., W. Burgin, A. Mombelli, Clin. Oral Implants Res. 12 (2001) 189–195.
[9] M. Quirynen, W. Teughels, Periodontology 2000 33 (2003) 119–128.
[10] S. Sumida, K. Ishihara, M. Kishi, K. Okuda, Int. J. Oral Maxillofac. Implants 17 (2002) 696–702.
[11] J.C. Wataha, J. Oral Rehabil. 23 (1996) 79–90.
[12] G. Nakazato, H. Tsuchiya, M. Sato, M. Yamauchi, Int. J. Oral Maxillofac. Implants. 4 (1989) 321–326.
[13] L.E. Wolinsky, P.M. De Camargo, J.C. Erard, M.G. Newman, Int. J. Oral Maxillofac. Implants 4 (1989) 27–31.
[14] D.R. Drake, J. Paul, J.C. Keller, Int. J. Oral Maxillofac. Implants 14 (1999) 226–232.
[15] Ä. Leonhardt, J. Olsson, G. Dahlén, J. Dent. Res. 74 (1995) 1607–1612.
[16] C.W. Berry, T.J. Moore, J.A. Safar, A.C. Henry, M.J. Wagner, Implant Dent. 1 (1992) 59–65.
[17] S. Bose, M. Roy, K. Das, A. Bandyopadhyay, Mater. J. Sci. Mater. Med. Suppl. 1 (2009) S19–S24.
[18] Y. Horiuchi, M. Horiuchi, T. Hanawa, K. Soma, Dent. Mater. J. 26 (2007) 924–929.
[19] V. Antoci Jr., C.S. Adams, J. Parvizi, P. Ducheyne, I.M. Shapiro, N.J. Hickok, Clin. Orthop. Relat. Res. 461 (2007) 81–87.
[20] P. Petrini, C.R. Arciola, I. Pezzali, S. Bozzini, L. Montanaro, M.C. Tanzi, P. Speziale, L. Visai, Int. J. Artif. Organs 29 (2006) 434–442.
[21] B. Del Curto, M.F. M.F. Brunella, C. Giordano, M.P. Pedeferri, V. Valtulina, L. Visai, A. Cigada, Int. J. Artif. Organs 28 (2005) 718–730.
[22] L. Yang, B.W. Sheldon, T.J. Webster, Biomaterials 30 (2009) 3458–3465.
[23] C.M. Stanford, Aust. Dent. J 53 (Suppl. 1) (2008) S26–S33.
[24] J.A. Parker, X.F. Walboomers, J.F. Von den Hoff, J.C. Maltha, J.A. Jansen, J. Biomed. Mater. Res. 61 (2002) 91–98.
[25] L. Visai, L. Rimondini, C. Giordano, B. Del Curto, M.S. Sbarra, R. Franchini, C. Della Valle, R. Chiesa, Appl. Biomater. Biomech. 6 (2008) 170–177.
[26] C. Yao, T.J. Webster, J. Nanosci. Nanotechnol. 6 (2006) 2682–2692.
[27] K.H. Kim, N. Ramaswamy, Dent. Mater. J. 28 (2009) 20–36.
[28] U. Diebold, Surf. Sci. Rep. 48 (2003) 53–229.
[29] A. Mills, S. Le Hunte, J. Photochem. Photobiol. A: Chem. 108 (1997) 1–35.
[30] T. Matsunaga, R. Tomoda, T. Nakajima, H. Wake, FEMS Microbiol. Lett. 29 (1985) 211–214.
[31] Y. Kikuchi, K. Sunada, T. Iyoda, K. Hashimoto, A. Fujishima, J. Photochem. Photobiol. A: Chem. 106 (1997) 51–56.
[32] N. Suketa, T. Sawase, H. Kitaura, M. Naito, K. Baba, K. Nakayama, A. Wennerberg, M. Atsuta, Clin. Implant Dent. Relat. Res. 7 (2005) 105–111.
[33] F.r. Marciano, D.A. Lima-Oliveira, N.S. Da-Silva, A.V. Diniz, E.J. Corat, V.J. Trava-Airoldi, J. Colloid. Interface Sci. 340 (2009) 87–92.
[34] C. Giordano, E. Sandrini, B. Del Curto, E. Signorelli, G. Rondelli, L. Di Silvio, J. Appl. Biomater. Biomech. 2 (2004) 35–44.
[37] G.D. Christensen, W.A. Simpson, J.J. Younger, L.M. Baddour, F.F. Barrett, D.M. Melton, E.H. Beachey, J. Clin. Microbiol. 22 (1985) 996–1006.
[38] M. Schuler, G.R. Owen, D.W. Hamilton, M. de Wild, M. Textor, D.M. Brunette, S.G. Tosatti, Biomaterials 27 (2006) 4003–4015.
[39] F. Chen, X. Yang, F. XuF, Q. Wu, Y. Zhang, Environ. Sci. Technol. 43 (2009) 1180–1184.
[40] S. Imazato, Dent. Mater. 28 (2009) 11–19.
[41] Y. Horiuchi, M. Horiuchi, T. Hanawa, K. Soma, Dent. Mater. 26 (2007) 924–929.
[42] E. Verné, M. Miola, C. Vitale Brovarone, M. Cannas, S. Gatti, G. Fucale, G. Maina, A. Massé, S. Di Nunzio, J. Mater. Sci. Mater. Med. 20 (2009) 733–740.
[43] L. Le Gúehennec, A. Soueidan, P. Layrolle, Y. Amouriq, Dent. Mater. 23 (2007) 844–854.
[44] A. Wennerberg, T. Albrektsson, Clin. Oral Implants Res. 20 (2009) 172–184.
[45] K. Dasa, S. Bosea, A. Bandyopadhya, Acta Biomater. 3 (2007) 573–585.
[46] J. Verran, K. Whitehead, Int. J. Artif. Organs 28 (2005) 1138–1145.
[47] Y.H. Tsuang, J.S. Sun, C.Y. Huang, C.H. Lu, W.H. Chang, C.C. Wang, Artif. Organs 32 (2008) 167–174.
[48] Y. Oka, W.C. Kim, T. Yoshida, T. Hirashima, H. Mouri, H. Urade, Y. Itoh, T. Kubo, J. Biomed. Mater. Res. B: Appl. Biomater. 86B (2008) 530–540.
[49] L. Visai, C.R. Arciola, G. Pietrocola, S. Rindi, P. Olivero, P. Speziale, Int. J. Artif. Organs 30 (2007) 813–819.
[50] P.S. Stewart, J.W. Costerton, Lancet 358 (2001) 135–138.
[51] A. Scarano, A. Piattelli, A. Polimeni, D. Di Iorio, F. Carinci, J. Periodontol. 81 (2010) 1466–1471.
[52] X.X. Wang, S. Hayakawa, K. Tsuru, A. Osaka, J. Biomed. Mater. Res. 52 (2000) 171–176.