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Bone tissue response to titanium implant surfaces modified with carboxylate and sulfonate groups

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Abstract The present study assessed in vivo new bone formation around titanium alloy implants chemically grafted with macromolecules bearing ionic sulfonate and/or carboxylate groups. Unmodified and grafted Ti–6Al–4V exhibiting either 100% carboxylate, or 100% sulfonate, or both carboxylate and sulfonate groups in the percent of 50/50 and 80/20 were bilaterally implanted into rabbit femoral condyle. Neither toxicity nor inflammation were observed for all implants tested. After 4 weeks, peri-implant new bone formation varied as a function of the chemical composition of the titanium surfaces. The percent bone-implant contact (BIC) was the lowest (13.4 ± 6.3%) for the implants modified with grafted carboxylate only. The value of BIC on the implants with 20% sulfonate (24.6 ± 5.2%) was significantly ($P < 0.05$) lower than that observed on 100% sulfonate (38.2 ± 13.2%) surfaces. After both 4 and 12 weeks post-implantation, the BIC value for implants with more than 50% sulfonate was similar to that obtained with the unmodified Ti–6Al–4V. The grafted titanium alloy exhibiting either 100% sulfonate or carboxylate and sulfonate (50% each) groups promoted bone formation. Such materials are of clinical interest because, they do not promote bacteria adhesion but, they support new bone formation, a condition which can lead to osseointegration of bone implants while preventing peri-implant infections.

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1 Introduction

Because of their biocompatibility and biomechanical properties, titanium and its alloys are extensively used in oral implantology for the rehabilitation of partially and fully edentulous patients. Despite routine clinical use, failure of implant integration still occurs due, among other reasons, to either unsatisfactory response of the tissue surrounding the implant surface and or to susceptibility to bacteria-related peri-implantitis [1, 2]. In the aforementioned cases, events at the microenvironment surrounding such implants involve cell interactions with material surfaces. Optimal clinical outcomes require absence of bacteria interaction (such as, adhesion, colonization, etc.) but support of bone cell functions pertinent to new tissue formation.

This clinical need has motivated research which addressed these challenging problems from various perspectives (clinical, pharmacological, etc.). In terms of biomaterials, chemical modification of existing as well as development of new materials which prevent bacterial

adhesion and colonization have been explored in order to withstand implant-related infection [3, 4].

For example, material-surface chemistry modification strategies to reduce bacterial adhesion have been explored. These endeavors included biomaterial surfaces modified either with ion (Ca+, N+, F+) implantation, plating (TiN, alumina), ion (Ag, Sn, Zn, Pt) beam mixing [5], polycationic groups [6, 7] or polyelectrolyte multilayers of acid hyaluronic and chitosan [8]. Another approach to minimize bacterial adhesion was to modify implant material surfaces with bioactive polymers bearing sulfonate and carboxylate groups. When present, by co-polymerisation or by grafting, on select polymers such as poly(methyl methacrylate) (PMMA) or silicone matrices such ionic groups inhibited *S. aureus* adhesion in vitro [9, 10] and in vivo [11]. Specifically, fewer (in the range of 40–90%) *S. aureus* bacteria adhered on silicone prostheses coated with C- and S-groups [11]. In additions, S groups grafted by chemical oxidation and direct radical polymerization on Ti reduced *P. gingivalis* adhesion [12].

Materials modified with polymers functionalised by carboxylate/sulfonate grafting are attractive candidates for implantation because they inhibit bacterial adhesion [9, 10] but, by interacting with adhesive proteins such as fibronectin [13], support fibroblast [14] as well as osteoblast functions (such as adhesion, proliferation, and maintenace of osteoblastic phenotype) pertinent to new bone formation [10] in vitro. To date, the potential for osteointegration of these modified materials has not been determined. The aim of the present in vivo project was, therefore, to use the rabbit femur (a well documented model) in the investigation of osteointegration [15] and of the host-tissue response around titanium alloy implants with surfaces grafted by polymers bearing carboxylate/sulfonate groups.

2 Materials and methods

2.1 Implant surface modification

Cylinders (5 mm in diameter and 6 mm in height) of medical grade titanium alloy (Ti–6Al–4V) (Spine Next, Bordeaux, France) were used in this study. Each implant material surface was grafted by anionic polymers bearing carboxylate and/or sulfonate groups. Anionic groups were grafted on Ti–6Al–4V following established and published procedures [16, 17]. Success of grafting on the titanium surfaces of interest to the present study were tested by toluidine blue assay and infra-red spectroscopy (HATR-FTIR). Based on their carboxylate and sulfonate ratio, the modified implants were classified into four test groups: C0/S100, C50/S50, C80/S20, C100/S0, where the numbers refer to the percentage of carboxylate and sulfonate, respectively. The control group consisted of the unmodified titanium alloy. Prior to experiments with animals, all implants were washed in phosphate buffered saline (PBS), degreased by immersion in 70% alcohol, sonicated for 10 min, and then sterilized by autoclaving.

2.2 Animals

Four-month-old (average weight of 3.5 kg) male New Zealand rabbits (Segav, Saint-Mars d'Egrenne, France) were used in the study. These animals were housed individually in metal hutches in an environment (ambient temperature of 21°C and 50% air humidity) that met the requirements of the *European Guidelines for Care and Use of Laboratory Animals* (Directive du conseil 24.11.1986, 86/609/CEE). Artificial lighting was used in the animal housing facility to maintain a normal day/night biological rhythm for the duration of the study. The animals were fed with water and commercial (Pietrement, Sainte Colombe, France) food concentrates ad libitum.

2.3 Surgical procedure

The rabbits were anesthetized via intramuscular injection of 0.5 mg/kg Diazepan (Valium®, Roche, Basel, Switzerland), 0.25 mg/kg metedomidine hydrochloride (Domitor®, Virbac, France), and 100 mg/kg ketamine hydrochloride (Ketalar 500®, Pfizer, France). The animals were prepared for surgery, shaved and disinfected; both lower limbs sites were draped. Then, a longitudinal skin incision was made to expose the distal lateral aspect of each femoral condyle. A cylindrical cavity was created in the lateral condyle in a stepwise fashion using color-coded, 6-mm-length and 1.5–5.2 mm diameter surgical drills (IDI system, Paris, France). These cavities were thoroughly rinsed with isotonic saline to remove bone fragments. Implants were placed in the cavities as described in Sect. 2.5. Each wound was closed in three successive layers (ligaments, soft tissue, and skin), and the exterior surface of the surgical site was disinfected.

2.4 Postsurgery animal care and euthanasia

All animals received intramuscular injections of 0.2 mg/kg metoxicamn (Metakam® Boehringer Ingelheim Vetmedica GmbH, Germany) to relieve pain during the postoperative 24-h period. Prophylactic antibacterial treatment, consisting of sulfadimethoxine trimethoprime at 25 mg/kg (Copylap® Biové, France), was also administered for 5 days after surgery. Post-surgery and during the study, the animals were allowed to walk. The rabbits were euthanized either at 4 or 12 weeks post-implantation using an overdose of pentobarbital. The femoral condyles were excised and cleared of the surrounding soft tissue. All bone

specimens were prepared for subsequent histological analysis as described in Sect. 2.7.

2.5 Experimental design

Each one of 24 rabbits was operated bilaterally. Forty-eight defects were assigned randomly to either one of the test or control groups. Six implants per group were analysed 4-weeks post-implantation. Six implants for two test groups, (specifically, C50/S50 and C0/S100) and controls were analysed after 12-weeks post-implantation.

2.6 Micro X-ray analysis

In order to determine the orientation of the sections for histological analysis, the excised specimens were rinsed in water, dehydrated in ethanol, and X-rayed using a *Faxitron*® (Faxitron X-Ray LLC, Lincolnshire, IL; 10 s exposure at 26 kV). Sections (500-μm thick) were micro-x rayed; micrographs were obtained before histological analysis of each specimen.

2.7 Histology

An histological procedure for non-demineralized bone was used for all excised tissue specimens. Each bone specimen was fixed in 10% phosphate-buffered formalin, rinsed in water, dehydrated in ethanol, cleared in xylene, and embedded in methyl methacrylate. Radiographs were taken to ensure appropriate defect orientation for subsequent histologic sectioning. The femoral condyles were sectioned perpendicular to the long axis of the implant using a circular water-cooled diamond saw (Microcut, Brot®, France). Each section was then grounded down to a thickness of about 70 μm, using an Exact Grinding System (Exact Aparatebau GmbH Norderstedt, Germany). The surfaces of these preparations were stained with Stevenels' blue and van Gieson picro-fuschin for subsequent standard light microscopy and/or histomorphometric analysis.

2.8 Histomorphometry

Three sections per condyle were histomorphometrically analyzed. Two parameters, specifically, the percent of bone tissue in contact with each implant (BIC) and the percent of mineralized bone area (MBA) in the circumferential zone (50 μm) around each implant were determined. Measurements were made using custom-made software in conjunction with an image processing system consisting of a microscope (DBMR Leica, Leica GmbH, Germany) and a video-camera (CUE-2 Olympus Q1A0150, *Olympus* Opticals Europe, Hamburg, Germany). BIC was calculated from the sum of the regions where bone was in contact with each implant. Briefly, the image (magnification: 20×) was digitized, a circle was drawn at the implant perimeter, then bone in direct contact with selected arcs was identified and the corresponding angle at the center of the circle was measured (in degrees). Bone implant contact (BIC) was expressed as a percentage out of 360°. In order to calculate the MBA, each histology image (magnification: 20×) was digitized, and a ring (50 μm wide) was delineated around the perimeter of each implant. The fraction of this annular area which was covered by mineralized tissue, was measured and expressed as the percent of the total tissue area.

2.9 Statistical analysis

Numerical data were reported as mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA) and Fisher's PLSD test using Statview 5.0 statistics software (SAS Institute, Berkeley, California). Significance was defined as a P value of less than 0.05.

3 Results

3.1 Animal morbidity and mortality

All rabbits were ambulatory within 3 h after surgery. Upon implant excision at the time of sacrifice, a fracture on one femur was observed; this animal was removed from the study and replaced by another one. No infection was observed in all animals for the duration of the study.

3.2 Micro X-ray results

In the radiografts, the implants were identified as radiopaque areas within the femora. At 4 weeks post-implantation, no radiolucent areas were observed around all implants tested (Fig. 1a–e). Moreover, there were no signs of osteolysis around the implants. At 4-weeks post-implantation, new bone formation in contact with all implants was observed. Trabecular bone organisation in contact with the implant surface was more compact and, thus, clearly visible compared to that observed in the epiphysis area; similar results were observed around the control and modified titanium-alloy implants (Fig. 1a–e).

Twelve weeks post-implantation, trabecular rarefaction with large medullary lacunae was observed in the posterior area of the femur epiphyses at a distance of the implants surface (Fig. 1f–h). At that time, a ring of bone was in contact with the implant. These results provide radiological evidence of compact bone.

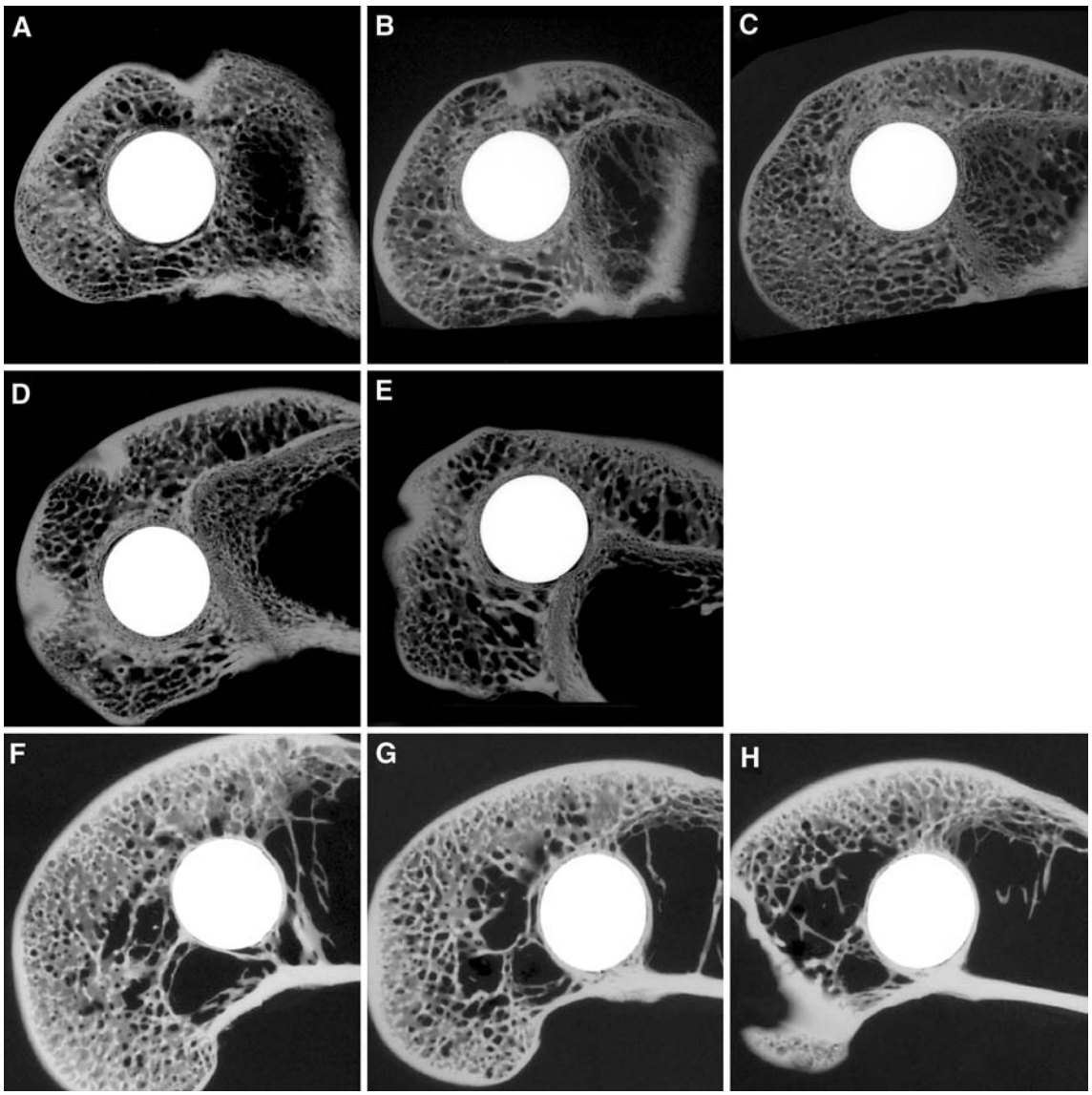

Fig. 1 Micro X-rays of rabbit femurs 4 and 12 weeks post-implantation. New bone surrounded the implant in all cases tested. **a** unmodified titanium; **b** C0/S100; **c** C50/S50; **d** C80/S20; **e** C100/S0; **f** unmodified titanium; **g** C0/S100; and **h** C50/S50. Specimens in **a–e** and **f–h** were excised 4 and 12 weeks post-implantation. C = Carboxylate, S = Sulfonate

3.3 Histologic observations

At 4 weeks post-implantation, no fibrous encapsulation of the implants was observed. Mineralized bone in direct apposition to the material surface was present around all implants tested (Fig. 2a–e). The implant surfaces were partially covered by bone. Both controls and implants with modified surfaces were surrounded by lamellar bone trabeculae with haversian canals lined by osteoblasts and contained many osteocytes. The non-mineralized tissue consisted of bone marrow, and included cells and blood vessels.

At 12 weeks post-implantation, the bone in direct contact with the control implant surface exhibited an organized structure (Fig. 3a–c). The histological aspects of these specimens were different than those obtained after 4 weeks of implantation. In the case of control implants, a continuous ring (approximately 220 μm thick) of bone, which was similar to cortical bone with regard to density and development of Haversian canals, was observed (Fig. 3a–c). This tissue was lined by osteoblasts (Fig. 3a, c), and was surrounded by bone marrow. In some areas of close contact with the implant surface, primary and secondary osteons were observed (Fig. 3b, c). This ring of mineralized bone tissue was surrounded by bone marrow and was often connected to the surrounding trabecular bone laterally. No differences in bone morphology were observed among the implant groups tested.

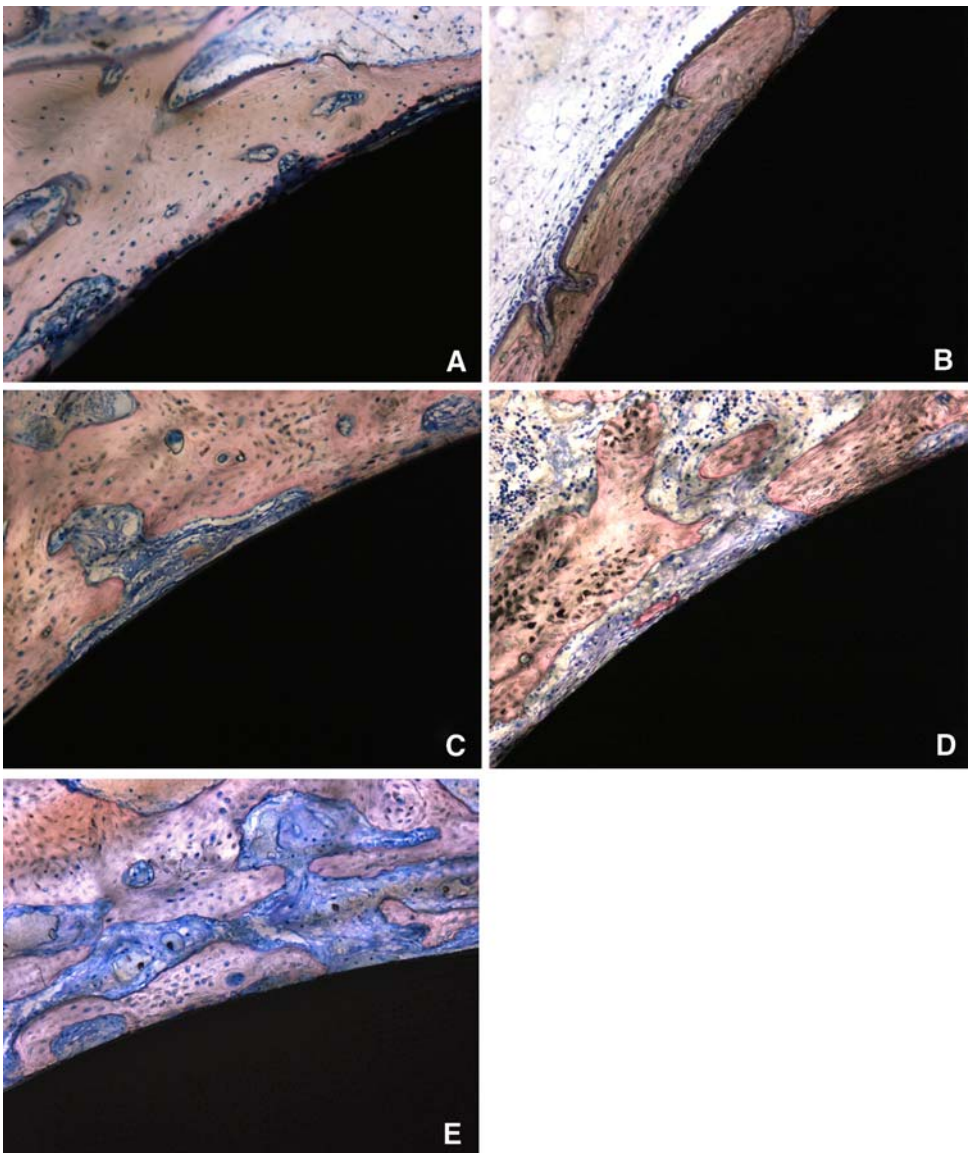

Fig. 2 Light micrographs of representative histology sections 4 weeks post-implantation. Bone tissue was present around all implants of interest to the present study. **a** unmodified titanium; **b–e** titanium whose surfaces were modified by immobilizing various amounts of C and S: specifically, C0/S100 (**b**); C50/S50 (**c**); (C80/S20) (**d**) and C100/S0 (**e**). Magnification = 10× for **a–e**. The black region on each frame is part of the area that had been occupied by the implant in vivo. Stains: Stevenels' blue (for visualization of the cell nuclei) and van Gieson picro-fuschin (for staining the bone tissue). C = Carboxylate, S = Sulfonate

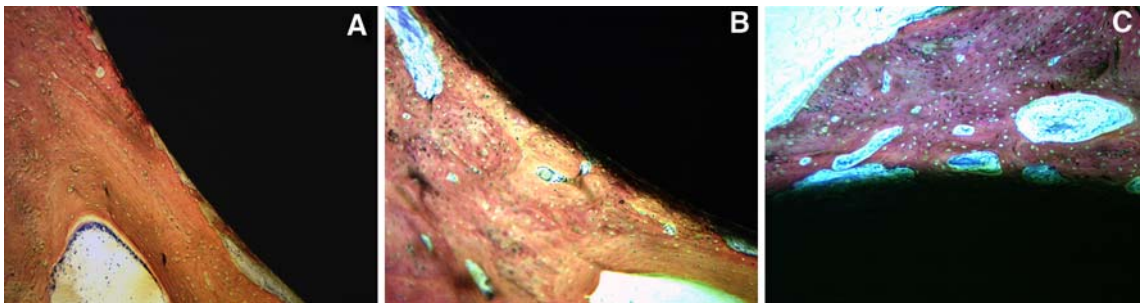

Fig. 3 Light micrographs of the bone/implant interface 12 weeks post-implantation. Bone was present on all implant surfaces tested. Osteoid tissue was present at the bone surface in contact with the medullary spaces. **a** unmodified titanium; **b** C0/S100; **c** C50/S50; Magnification for **a–c** = ×10. Stains: Stevenels' blue (for visualization of the cell nuclei) and van Gieson picro-fuschin (for staining the bone tissue). C = Carboxylate, S = Sulfonate

3.4 Histomorphometric results

At 4 weeks post-implantation, the percent of BIC decreased with increased carboxylate content on the implant material surface (Fig. 4a). For implants with surfaces modified with 100% (C0/S100) and 50% sulfonate (C50/S50), the BIC was similar to that observed for controls (38 ± 13.2%, 26.4 ± 8.9 and 32.1 ± 17.7, respectively). The percent BIC on the C0/S100 was significantly ($P < 0.05$) higher than on the C80/S20 surfaces (38.2 ± 13.2% versus 24.6% ± 5.2%). The percent BIC on the C100/S0 surfaces was the lowest (13.4% ± 6.3%), and significantly different than that observed on the controls ($P < 0.05$), and on the C50/S50 ($P < 0.001$) surfaces (Fig. 3a). Moreover, the correlation between the percent sulfonate content on the modified titanium surfaces and BIC was linear ($P < 0.001$) (Fig. 4b).

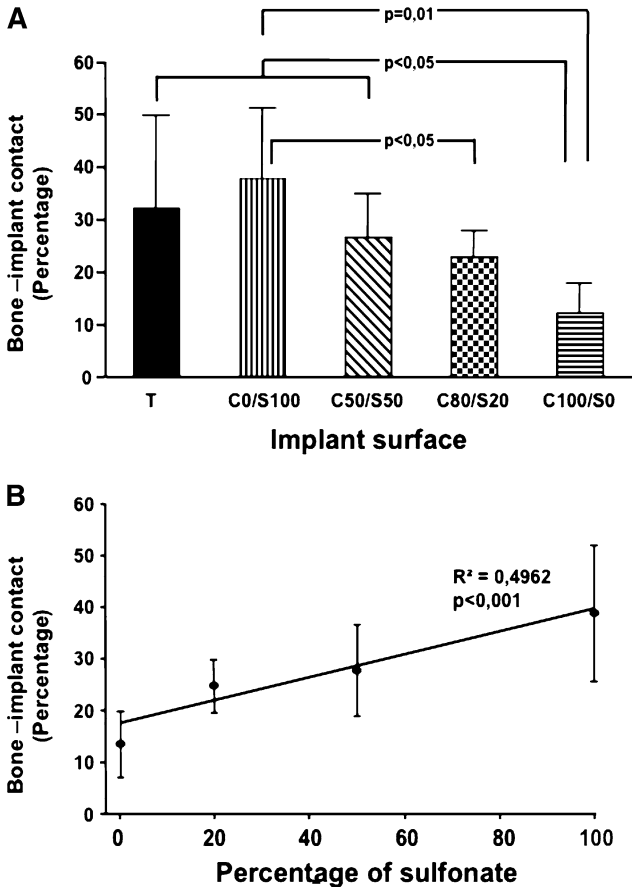

Fig. 4 **a** Bone-implant contact (BIC) on various titanium surfaces 4-weeks post-implantation. The BIC was related to the implant surface composition. Specifically, maximum percent BIC was obtained for S = 100% but decreased with percent sulfonate content; for example, the percent BIC was significantly lower for S ≤ 20%. S = sulfonate. **b** Relation between bone-implant contact (BIC) and percent sulfonate grafted on the titanium surface. The correlation between percent BIC and percent sulfonate content was linear and significant ($P < 0.001$). C = Carboxylate, S = Sulfonate

At 4 weeks post-implantation the percent of the mineralised bone area (MBA) in the zone within 50 μm around the implant decreased with increased carboxylate content on the implant material surface (Fig. 5a). The percent MBA around implants with 100% sulfonate (C0/S100) and 50% sulfonate (C50/S50) on their surfaces was similar to that observed around unmodified titanium (57.2 ± 13.3, 57.5 ± 4.2 and 51.7 ± 7.2, respectively). The percent MBA around the C80/S50 and C100/S0 surfaces was significantly ($P < 0.05$) lower than that observed around the unmodified and C0/S100 surfaces (Fig. 5a). The correlation between the BIC and MBA was linear $P < 0.001$). The percent MBA values within the annular areas 150 μm and 500 μm from the implant perimeter were similar of all groups tested (data not shown).

At 12 weeks post-implantation, the amount (approximately 27%) of BIC was similar to that observed around the control implants after 4 weeks of implantation (Fig. 6). In addition, 12 weeks post-implantation the percent BIC, as well as the percent MBA, on S50/C50 was similar to that observed on the unmodified titanium and on the C0/S100 surfaces (Fig. 5b).

4 Discussion

The present in vivo study is the first to determine the osteointegration effect of anionic polymers bearing carboxylate/sulfonate groups grafted on Ti–6Al–4V implants. These ionic groups are of clinical interest because, when incorporated in the macromolecular chains of polymers, they induce reduced adhesion of *S. aureus* [9, 11] and when

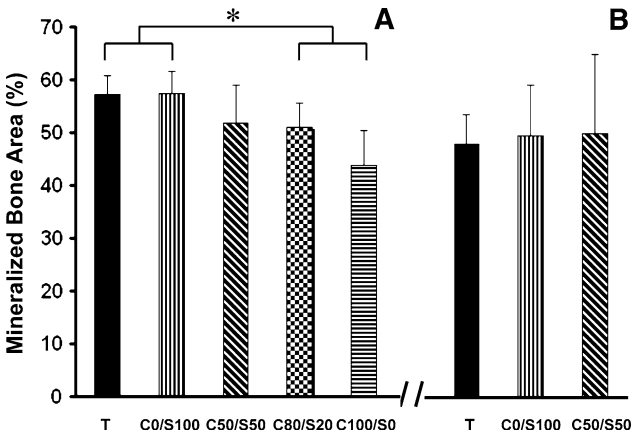

Fig. 5 Mineralized bone area (MBA) around titanium surfaces and around chemically modified titanium surfaces. **a** 4-weeks post-implantation; maximum percent MBA was observed for S = 100%. It was significantly ($P < 0.001$) lower for S = 20%. C = carboxylate; S = sulfonate. **b** 12-weeks post-implantation. The percent MBA on C50/S50 was similar to that observed on the C0/S100 and on the unmodified titanium implants

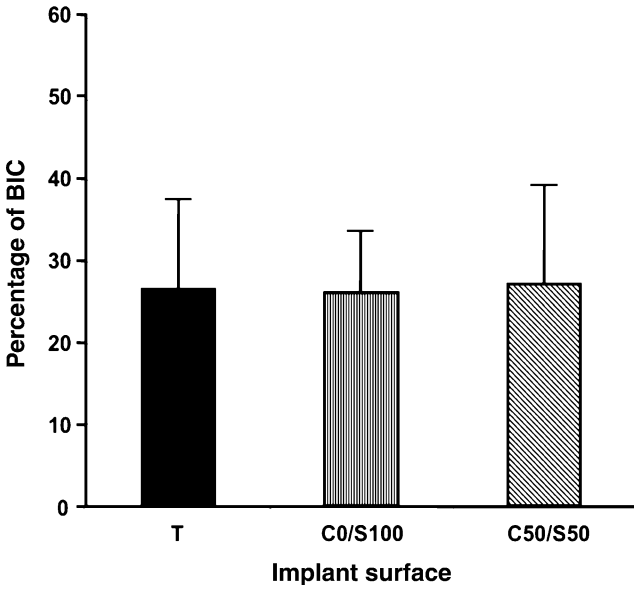

Fig. 6 Bone-implant contact (BIC) on titanium surfaces and on chemically modified titanium surfaces 12-weeks post-implantation. The percent BIC on C50/S50 was similar to that observed on the unmodified titanium and on the C0/S100 titanium implants

grafted on Ti reduce adhesion of *P. gingivalis* [12]; these bacteria are implicated in peri-implantitis and other conditions whose treatment requires clinical intervention.

Ion grafting on titanium and/or Ti–6Al–4V surfaces, may, however, modify the chemical characteristics of these materials surface which modulate surrounding bone tissue response and, therefore, the clinical performance of such implants. Ion implantation, which modifies the titanium surface chemistry, energy, and/or topography, affects bone healing [18]. Supporting evidence has been provided by a number of studies; for example, modulation of bone formation around titanium surfaces modified with either magnesium [19], Ca^2 [20], CO [21] or fluoride [22] was reported in the literature. The presence of Ca^2 ions on machined, commercially-pure (c.p.) titanium surfaces implanted in the rabbit femur [20] and of CO ions on either c.p. titanium or Ti–6Al–4V implanted in the rabbit femur and tibia [21] promoted bone formation. In addition, fluoride ions on grit-blasted c.p. titanium surfaces inserted in the rat tibia promoted interfacial bone formation [22] while magnesium on c.p. titanium was associated with increased bone formation in rabbit tibiae [19].

The results obtained in the present study provided evidence that BIC varied as a function of the carboxylate/sulfonate ratio on the titanium surfaces tested; specifically, the carboxylate ionic groups affected bone healing around implants. Since, in the present study, the surface of carboxylate/sulfonate-modified Ti–6Al–4V may have been coarsened by the ion implantation grafting process, the influence of not only surface composition but also surface roughness should be considered when evaluating the effects of modified material surfaces on bone formation around such implants. Since decreased BIC was observed only on surfaces with high carboxylate content, either the chemistry or topography or both aspects of those surfaces may be responsible for the observed outcomes.

Histomorphometric analysis of bone tissue in contact with the various surfaces tested in the present study demonstrated that increasing (up to 50%) the carboxylate content on the functionalized surfaces resulted in significant ($P < 0.05$) decrease of BIC 4 weeks post-implantation (Fig. 4a). The mechanisms underlying the observed decreased bone/implant contact with increasing carboxylate content on Ti–6Al–4V are still not known. Various implant material surface properties control both protein adsorption and thus subsequent cell functions (reviewed in Bagno and Di Bello [23]). The ionic groups grafted on material surfaces may further modulate some, or induce additional, such material surface properties that consequently affect cell functions [5]. In this respect, the observed effects of the carboxylate groups grafted on Ti–6Al–4V could be due to the type, amount and/or conformation of proteins adsorbed on those implant material surfaces in vivo; this outcome could modulate subsequent adhesion and/or other functions of osteogenic, blood and other cell types present at the implant microenvironment during the initial stages of peri-implant endosseous healing and, therefore, subsequent interactions of the surrounding tissues. For instance, heparin-binding domains of the fibronectin exhibited by adsorbed on PMMA-based polymers (with a ratio of $COO^-/COO^- + SO_3^-$ around 0.6) are different to that exhibited on unmodified PMMA [13]. This difference in the fibronectin conformation may explain (at least in part) sub-optimal spreading of fibroblasts in vitro [13], the slower proliferation of fibroblastes in vitro [14], and in the decreased osteoblast proliferation [10] observed on these modified PMMA-based polymers.

A second explanation for the decreased bone/implant contact with increasing carboxylate pertains to the effects of carboxylate ions on transient fibrin-based structures of blood clots. Studies reported changes in fibrin binding on titanium surfaces and in the migration of osteogenic cells through the three dimensional matrix of fibrin clots [24]. Ion implantation may alter the Ti–6Al–4V surface thrombogenic properties. For instance, a fluoride ion modification augmented the titanium thrombogenic properties resulting in a less dense fibrin clot that promoted both fibrinolysis and cell migration during early wound healing [25]. In this respect, differences in both the number and function of cells (such as neutrophils, macrophages, etc.) at the implant material surfaces containing carboxylate (up to 50%) may also account for the decreased bone-implant contact observed in the present study 4 weeks

post-implantation. Elucidation of the cellular and molecular mechanisms behind the effect of high content of carboxylate (as well as high contents of sulfonate on the osteointegration of Ti–6Al–4V requires further investigation, which was out of the scope of the present study.

An interesting finding of the present study was that bone healing around Ti–6Al–4V surfaces with high (>50%) sulfonate content, which have anti-adhesive bacterial properties when grafted on Ti [12], exhibited similar BIC as that observed around unmodified Ti–6Al–4V at 4 and 12 weeks post-implantation. In the present study, bone had covered about 32% of the unmodified Ti–6Al–4V implant surface 4 weeks post-implantation; during this time period, complete bone formation occurred in the rabbit femoral model [15]. Similar percentage of contact between bone and Ti or Ti–6Al-surface (BIC) was reported by other studies which also used the rabbit experimental model [15, 26]. Due to differences in the experimental conditions and in the assessement parameters used by the aforementioned studies, however, it is not possible to directly compare the results of the present study with those of other histomorphometric studies which used either titanium or Ti–6Al–4V.

In the present study, after 12 weeks, the amount of BIC for the two surfaces with high (specifically, 100 and 50%) sulfonate content tested, was similar to that obtained 4 weeks post-implantation. Although at best rabbit femoral condyles partially reflect the situation in the jaw, similar trends were reported by other researchers who used Ti machined implants in dog mandibles and examined bone formation either 3 and 8 weeks [27] or to 8 and 12 weeks post-implantation [28]. The fact that the BIC values for surfaces with 50 and 100% sulfonate content, were similar at longer healing times indicate a long-term bone tissue stability around the implant with the sulfonate-modified surfaces. At 12 weeks post-implantation, a trabecular rarefaction was observed, in particular in the distal and posterior location of the implants, independently on their surface modification. It may be associated with the continued bone remodelling, secondary to the implant insertion and/or to the normal bone loss of aging. The fact that the rarefaction observed is limited to a specific area suggests that stress distribution could be a critical factor, but its contribution to the bone remodelling remains to be answered.

5 Conclusion

The carboxylate/sulfonate composition on Ti–6Al–4V material surfaces affected bone formation around such implants in rabbit femurs. BIC was similar to that observed on unmodified surfaces only when the sulfonate content was higher than 50% and remained the same for longer (specifically, 12 weeks) implantation times indicating long-term stability of the bone around those implants. These observations provide evidence that modifications of implant material surfaces with sulfonate content above 50% are non-toxic, biocompatible, and osteointegrable. For these reasons, only sulfonate, which is both not vulnerable to bacterial infection and supports new bone formation, should be grafted on Ti–6Al–4V surfaces used for orthopedic and dental implants.

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