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Evaluation in vitro and in vivo of biomimetic hydroxyapatite coated on titanium dental implants

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Abstract

Among several materials used as dental implants, metals present relatively high tensile strengths. Although metals are biotolerable, they do not adhere to bone tissues. On the other hand, bioactive ceramics are known to chemically bind to bone tissues, but they are not enough mechanically resistant to tension stresses. To overcome this drawback, biotolerable metals can be coated with bioactive ceramics. Various methods can be employed for coating ceramic layers on metal substrates, among them ion sputtering, plasma spray, sol–gel, electrodeposition and a biomimetic process [E.C.S. Rigo, L.C. Oliveira, L.A. Santos, A.O. Boschi, R.G. Carrodeguas. Implantes metálicos recobertos com hidroxiapatita. Revista de Engenharia Biomédica, vol. 15 (1999), números 1–2, 21–29. Rio de Janeiro].

The aim of this work was to study the effect of the substitution of G glass, employed in the conventional biomimetic method during the nucleation stage, by a solution of sodium silicate (SS) on the chemical and morphological characteristics, and the adhesion of biomimetic coatings deposited on Ti implants. The obtained coatings were analyzed by diffuse reflectance FTIR spectroscopy (DRIFT) and scanning electron microscopy (SEM). Titanium implants were immersed in synthetic body fluid (SBF) and SS. All implants were left inside an incubator at 37 °C for 7 days, followed by immersion in 1.5 SBF and taken back to the incubator for additional 6 days at 37 °C. The 1.5 SBF were refreshed every 2 days. At the end of the treatment, the implants were washed in distilled and deionized water and dried at room temperature. To check the osseointegration, titanium implants coated with biomimetic method were inserted in rabbit's tibia, remaining there for 8 weeks. During the healing period, polyfluorochrome sequential labeling was inoculated in the rabbits to determine the period of bone remodeling. Results from DRIFT and SEM showed that, for all processing variants employed, a HA coating was always obtained on the Ti implants. Besides, G glass employed during the nucleation stage can be effectively substituted by a sodium silicate solution according to these results. The presence of implants stimulated the bone growth in the medullar region and the use of polyfluorochrome sequential labeling allowed the identification of the period of bone deposition and bone reorganization.

Keywords: Coating; Biomimetic; Ti implants; Hydroxyapatite; Osteointegration

1. Introduction

Commercially pure titanium and some titanium alloys including Ti6Al4V have been widely used in the manufacturing of dental and orthopaedic implants. Although bulk properties dictate the mechanical properties of biomaterials, tissue-biomaterial processes are surface phenomena and they are governed by surface properties. However, bioactivity of titanium surfaces is not high enough to induce the direct growth of the bone tissue and good bone fixation takes several months. Modifications of metal surfaces often are employed as a mean of controlling tissue-titanium interactions and shortening the time bone fixation [2].

The application of hydroxyapatite (HA) coatings on metallic implant devices offers the possibility of combining the strength of the metals with the bioactivity of the ceramics. Many different techniques have been used for the preparation

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of HA coatings among them ion sputtering, plasma spray, sol–gel, electrodeposition and a biomimetic process [1].

There is increasing interest in the low temperature, biomimetic preparation of coatings on implanted materials [3,4]. Recently, calcium phosphate materials have been grown biomimetically over different surface-modified substrates in order to gain insight into how to develop biomaterials with tailored surface properties that can initiate calcium phosphate deposition when implanted into body [3,4].

HA coatings can be deposited on the implants by the immersion in synthetic body fluid (SBF) and bioactivity glass at low temperatures. The glass releases Ca^{2+}, Na^{+} or K^{+} ions from its surfaces via an exchange with the H_3O^{+} ion in the SBF to form Si–OH or Ti–OH groups on their surfaces. Water molecules in the SBF then or simultaneously react with the Si–O–Si or Ti–O–Ti bond to form additional Si–OH or Ti–OH groups. The Si–OH and Ti–OH groups formed induce apatite nucleation, and released Ca^{2+}, Na^{+} or K^{+} ions accelerate apatite nucleation by increasing the ionic activity product of apatite in the fluid [2,3]. Once the apatite nuclei are formed, they can grow spontaneously by consuming the calcium and phosphate ions in the surrounding fluid because the body fluid is highly supersaturated with respect to the apatite.

The aim of this work was to study the effect of the substitution of G glass, employed in the conventional biomimetic method during the nucleation stage, by a solution of sodium silicate (SS) on the chemical and morphological characteristics, and the adhesion of biomimetic coatings deposited on Ti implants.

2. Experimental procedure

2.1. Biomimetic coating

The solutions utilized were SBF, 1.5 SBF and sodium silicate solution (SS) as another nucleating agent. They were prepared by dissolution of NaCl, KCl, K_2HPO_4, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $NaHCO_3$, Na_2SO_4 and $(Na_2O) \cdot SiO_2$ all of analytical purity in destilled and deionized water. The pH of all solutions was adjusted to 7.25 at 37 °C with 1 N HCl and tris(hidroxymetil)aminomethane. The solutions were kept in closed polyetylene containers.

Titanium implants were placed in a polyetylene recipient and were immersed in SBF and SS. All the substrates in their respective recipient were put in an incubator at 37 °C for 7 days. After this incubation period, the implants were immersed in destilled and deionized water and finally dried at room temperature.

Each treated implants were re-immersed in 1.5 SBF and put back in the incubator for more 6 days at 37 °C. The 1.5 SBF were renewed every 2 days. At the end of the treatment, the substrates were washed again in distilled and deionized water and dried at room temperature [5].

2.2. Operative procedure

Titanium implants with biomimetic coating were inserted in rabbit's tibia and remained for eight weeks. This study follows the Rules of the Ethics Committee in Animal and Research of Institute of Biomedical Sciences, University of Sao Paulo. Four female rabbits (*Oryctolagus cunniculus*) with an average weight of 3 kg were individually housed and kept in a 12-h light/dark cycle in a temperature-controlled (21 °C) designated animal room. After a period of housing, rabbits received 16 implants. According to the surgical plan two implants were inserted in the proximal margin of each tibia under general anesthesia and antibiotic protection. The surgical protocol was the same for all the animals under general anesthesia (intramuscular injection of ketamine 20 mg/kg). The medial skin of both legs was shaved, antisepsis was performed, and local anesthesia was supplied with 1:100,000-epinephrine vasoconstrictor. A full flap was made in the area near the surgical site and the implants were inserted in the proximal epiphysis of the tibias under saline irrigation. After the insertion, the flaps were carefully closed with a silk thread suture (Ethicon—Johnson's and Johnson's) [6].

2.3. Polyfluorochrome sequential label

For evaluation of the bone remodeling during the healing was carried out polyfluorochrome sequential labeling introduced by Rahn [7]. This technique allows marking the period of bone remodeled in agreement with the different colors of the markers. Subcutaneous injection of tetracycline, alizarin and calcein markers started in the second week and was repeated every 7 days, as described in Table 1. These doses were dissolved in saline solution together a solution lid of $NaHCO_3$.

2.4. Section preparation

After a healing period of 8 weeks, the animals were sacrificed with an overdose of pentobarbital. The tibias were dissected and bone blocks containing the implants were removed and maintained in a 4% neutral buffered formalin solution. After the fixation, the samples remained in running water for 12 h and dehydrated in different solutions of

Table 1
Sequence of the polyfluorochrome sequential labeling and days of application

Postop. (days)	Substance	Doses	Injection (mg/kg)
14	Tetracycline	60	3 g/100 ml+2 g $NaHCO_3$
21	Tetracycline	60	3 g/100 ml+2 g $NaHCO_3$
28	Alizarin	30	3 g/100 ml+2 g $NaHCO_3$
35	Alizarin	30	3 g/100 ml+2 g $NaHCO_3$
42	Calcein	10	3 g/100 ml+2 g $NaHCO_3$
49	Calcein	10	3 g/100 ml+2 g $NaHCO_3$
56	Exitus		

ethanol from 70° to 99° for 24–56 h in each solution. The samples were then embedded in a methacrylate solution (Tecnovit 7200 VCL, Heraeus Kuzer) with alcohol in gradual concentration from 30% to 100%. Samples were polymerized in blue light for 96 h. After the polymerization, they were processed according to the cutting-grinding technique [5–8]. The sections were separated in two groups: without staining and staining with Toluidine Blue. All the sections obtained were analyzed under light and fluorescent microscope (Nikkon Eclipse E 1000).

3. Results and discussion

3.1. Diffuse reflectance spectroscopy on the infrared (DRIFT)

The implants treated for 7 days, when they were re-immersed in 1.5 SBF (Fig. 1), the presence of well defined wide bands of 600 and 1040 cm^{-1} can be observed, all of them characteristic of the PO_4^{3-} ion vibration: of 870, 1410 and 1490 cm^{-1}, characteristic of the CO_3^{2-} ions; of 1650 cm^{-1}, characteristic of the H_2O; and a very wide band in the 3000–3600 cm^{-1} region, characteristic of the OH^- [9,10].

These results showed that the sodium silicate solution (SS) can be used to effectively replace the G glass as source of silicate ions. The mechanism of the apatite layer formation by the biomimetic method is the following: (1) silicate ions dissolved from the G glass are adsorbed on the substrate; (2) apatite nucleation occurs on the adsorbed silicate ions from the Ca^{2+} and PO_4^{3-} ions present in the SBF; (3) apatite nuclei grow directly on the substrate by a reaction with the solution, which is supersaturated to apatite [2]. The silicate ions adsorbed on the surface of the substrate produce silanol groups (Si–OH), which are accepted to be the responsible for the apatite nucleation action [2].

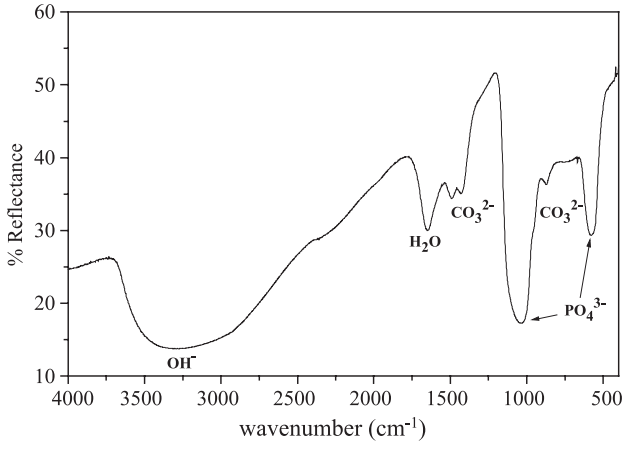

Fig. 1. Diffuse reflectance spectroscopy on the infrared (DRIFT) of the recovered Ti implants by biomimetic method, using as nucleating agent sodium silicate solution (SS) and re-immersion in 1.5 SBF.

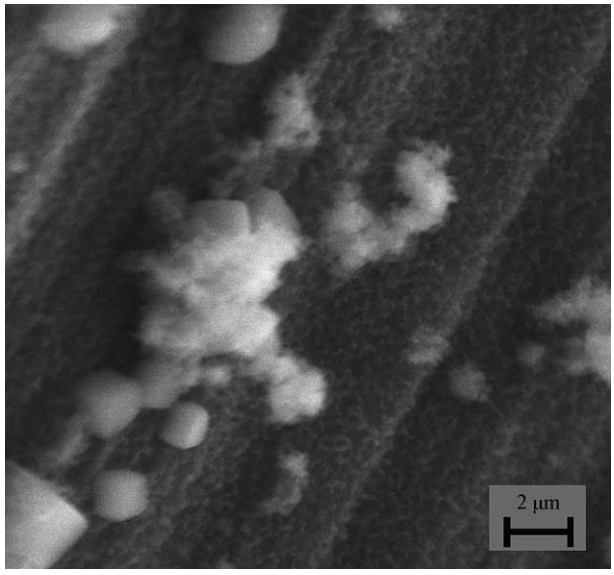

Fig. 2. SEM of the Ti implants recovered by biomimetic method, using as nucleating agents sodium silicate solution (SS) and re-immersion in 1.5 SBF.

3.2. Scanning electronic microscopy (SEM)

The Ti implants under study were analyzed by SEM and the resulting images are shown in Fig. 2.

For Ti implants treated for 7 days and re-immersed in 1.5 SBF, the formation of a compact dense layer can be observed and the formation of scarce globules on this layer, composed of primary agglomerated particles.

3.3. Osseointegration

Figs. 3 and 4 show pictures of the implant in the tibias' rabbit taken by a fluorescent microscope.

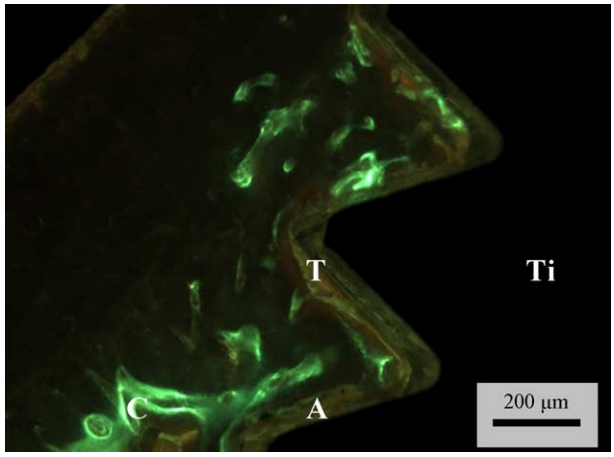

Fig. 3. Photomicrograph taken by a fluorescent microscope. The implant inserted in the rabbit's tibia is indicated in the figure (Ti). Remodeling in the medullar cavity and in the cortical region of bone tissues are also shown by the fluorescent markers: calcein (C), tetracycline (T) and alizarin (A).

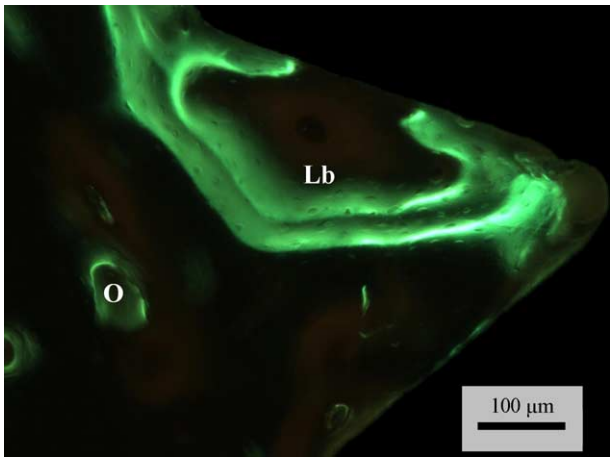

Fig. 4. Photomicrograph taken by a fluorescent microscope. Medullar cavity region. The presence of lamellae bone (Lb) deposition parallel to the implant and the formation of secondary osteons (O) are presented.

Figs. 3 and 4 show the pattern of bone tissues remodeling associated to the implant. A tissue response in the tibias' cortical in contact or near to the implant's surface is observed (Fig. 3). The first and the second label—tetracycline and alizarin—have a similar hue; tetracycline is light brown and alizarin is dark brown. They seem to be spread out. The bones marked as light brown and dark brown are the ones formed in the beginning and the middle of the bone remodeling, respectively. They are observed as apposition lamellar tissues and primary osteons in Fig. 4. The deposition of the third label—calcein—forming two lines at the interface bone/implant (Fig. 4) represents the maturation of the bone tissue. This label was injected at end of the healing period, which is represented by the secondary osteons that were formed (Fig. 4).

Figs. 5 and 6 show pictures of slices staining with the implant in the tibias' rabbit taken by light microscope.

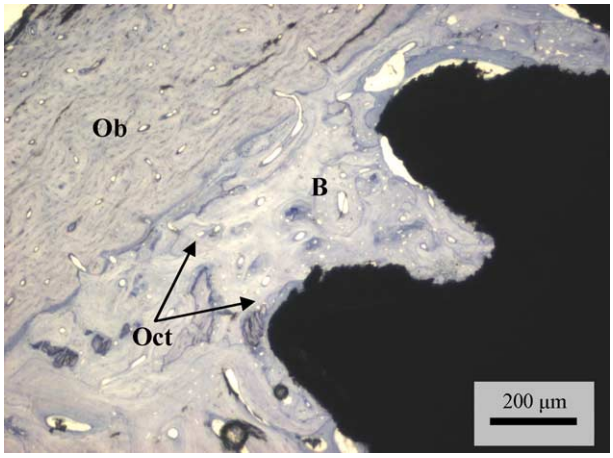

Fig. 5. Photomicrograph taken by a light microscope. Newly formed bone (B) in direct contact with the implant, osteocytes (Oct) cells and the interface with the old bone (Ob) was observed.

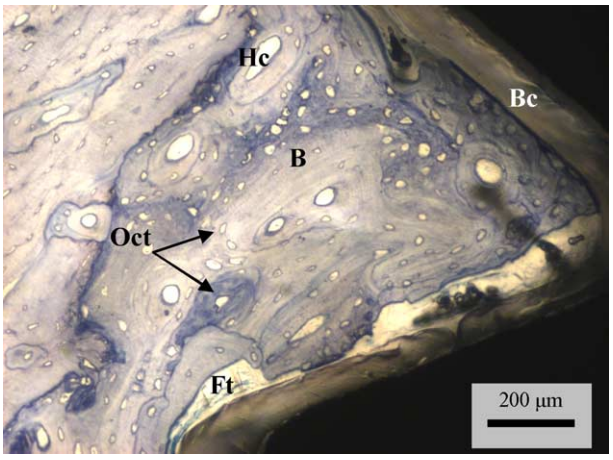

Fig. 6. Photomicrograph taken by a light microscope at a high magnification. Newly formed bone (B) in direct contact with the implant, osteocytes (Oct) cells, Haversian canal (Hc) and some fibrous tissues (Ft). The biomimetic coating (Bc) can be observed in the implant's surface.

Figs. 5 and 6 show the distribution of tissues in contact with the biomimetic coating implants. In general, the implants were surrounded mainly by bone tissues (Fig. 5), with some fibrous tissues present (Fig. 6).

During the bone repair, woven bone osteoblasts derived from bone marrow mesenchymal cells attach to the surface of the injured cortical bone (Fig. 5) to make a scaffold for the lamellar bone osteoblasts, which differentiate into immature lamellar bone osteocytes, then into mature lamellar osteocytes.

4. Conclusions

The formation of an apatitic coating on the Ti implants was always observed, which indicates that G glass can be effectively substituted by a solution of sodium silicate buffered to pH 7.25 and containing 100 ppm Si.

None at the rabbits showed any evidence of immune or inflammatory responses to the installed titanium implants.

After a healing period of 8 weeks, bone tissues remodeling associated to the implant were observed. The difference of deposition period of lamellar tissues (first and the second label—tetracycline and alizarin) and the maturation bone tissues (third label—calcein) were identified by polyfluorochrome sequential labeling. The implants were surrounded mainly by bone tissues, with few fibrous tissues in some regions.

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References

[1] E.C.S. Rigo, L.C. Oliveira, L.A. Santos, A.O. Boschi, R.G. Carrodeguas, Implantes metálicos recobertos com hidroxiapatita, Rev. Eng. Biomed. 15 (1–2) (1999) 21–29 (Rio de Janeiro).

[2] T. Kokubo, H.M. Kim, M. Kawashita, Novel bioactive materials with different mechanical properties, Biomaterials 24 (13) (2003) 2161–2175.

[3] Y. Abe, T. Kokubo, T. Yamamuro, Apatite coating on ceramics, metals and polymers utilising a biological process, J. Mater. Sci.: Mater. Med. 1 (1990) 233–238.

[4] C. Loty, J.M. Sautier, H. Boulekbache, T. Kokubo, H.M. Kim, N. Forest, In vitro bone formation on a bonelike apatite layer prepared by a biomimetic process on a bioactive glass-ceramic, J. Biomed. Mater. Res. 49 (2000) 423–434.

[5] E.C.S. Rigo, Recobrimento biomimético de implantes metálicos com hidroxiapatita. Tese de Doutorado, UFSCAR, São Carlos, 2001.

[6] B. König Jr., C. Lopes, Bone remodeling analysis after placement of dental implants using polyfluorochrome sequential labeling, Ann. Anat. 184 (2002) 241–244.

[7] B. Rahn, A. Die polychrome sequenzmarkierung, Freiburg i. Br., Habilitations-Schrift, 1976.

[8] K. Donath, G. Breuner, A method for the study of undecalcified bones and teeth with attached soft tissues, J. Oral Pathol. 11 (1982) 318–326.

[9] F. Barrère, P. Layrolle, C.A. Van Blitterswijk, K. De Groot, Biomimetic calcium phosphate coatings on Ti6Al4V: a crystal growth study of octacalcium phosphate and inhibition by Mg^{2+} and HCO_3^-, Bone 25 (2) (1999) 107S–111S.

[10] A. Stoch, W. Jastrzebski, A. Brozek, B. Trybalska, M. Cichocinska, E. Szarawara, FTIR absorption-reflection study of biomimetic growth of phosphates on titanium implants, J. Mol. Struct. 511–512 (1999) 287–294.