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Cell adhesion on different titanium-coated surfaces

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ABSTRACT

Titanium (Ti) is a biocompatible material, and calcium phosphate coating on titanium is commonly applied in order to obtain faster osseointegration around metallic implant. Osteoblast adhesion on three different Ti surfaces was evaluated. The investigated surfaces were commercially pure Ti (cp Ti), Ti coated with sodium titanate (Na-Ti), and Na-Ti followed by octacalcium phosphate coating (Ti-OCP) done by immersion in a calcium and phosphate-rich solution. The studied materials exhibited different morphology and composition. However, all the surfaces promoted cellular adhesion and showed cytocompatibility. No statistically significant difference was observed among the evaluated samples in relation to the number of cells.

Keywords: Titanium, octacalcium phosphate, cell adhesion, osteoblast.

1 INTRODUCTION

Metallic implants are necessary for hard tissue substitution, especially for load-bearing applications. Moreover, stainless steels and CrMo alloys, commercially pure titanium (cp Ti) and titanium alloys are largely used both in dentistry and in the orthopedic field. Several authors modified Ti surface aiming, for example, at roughness increase or ions liberation decrease.

Recently, several researchers were focused on understanding how the biomaterial surface can optimize bone formation or tissue binding [1-3]. The approach is to increase Ti surface integration with bone (osseointegration) by coating it with a bioactive material. Calcium phosphate (CaP) materials are the best choice, enabling the mechanical properties of the metallic titanium to join the osteconduction of bioactive materials. The coating layer may also enhance corrosion resistance, avoiding metallosis, which can drastically reduce the functional life of prosthesis.

Among the Ca-P biomaterials, hydroxyapatite (HA) is the phase most commonly studied for bone graft or coating. Octacalcium phosphate (OCP) is another CaP material that seems to play an important role in mineralization processes such as bone formation, enamel and dental calculi [4, 5]. Previous in vivo research have showed OCP biodegradability and its replacement by newly formed bone [6, 7]. Additionally, the OCP to HA conversion was observed in vivo and this process seems to stimulate the differentiation phenomenon of primary-osteoblastic cells [7]. A possible explanation for this could be related to a local increase in calcium concentration as well as favored adsorption of bone inductive proteins such as BMPs, bone morphogenic proteins [6]. Consequently, several researchers have developed OCP materials in various forms such as coating on metallic implant [1, 3] microscaffold [6] and granules for bone graft [8, 9].

The influence of biomaterials properties (chemical composition, topography and crystallinity) on cell response is reported in vitro and in vivo tests [10, 11]. From the crystallinity viewpoint, it has been showed that both poorly crystalline and amorphous calcium phosphates improve cell adhesion and proliferation as a consequence of their high solubility. Furthermore, an increase in the bone rate formation has been pointed out [12]. Accordingly, OCP can be an appropriate coating for metallic implants since it is more soluble than HA.

Thus, the purpose of the present study was to evaluate cell adhesion on different titanium surfaces: commercially pure Ti (cp Ti) and Ti coated either with a sodium titanate or an octacalcium phosphate layer.

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2 MATERIALS AND METHODS

2.1 Surfaces preparation

Sheets of commercial grade Ti (cp Ti) were used as substrate. Part of the samples was treated with 5M NaOH solution at 60 °C for 24 hours, followed by a thermal treatment at 600°C for 1h. This procedure is largely used by Kokubo's group on several substrates in order to activate metallic surface [13] and as a result, a sodium titanate layer coats the Ti surfaces (Na-Ti samples). The OCP coating was obtained on activated titanium sheets according to RESENDE *et al.* [3]. In brief, a less complex solution containing $NaHCO_3$, K_2HPO_4, $3H_2O$ and CaC_{l2} was prepared at 36ºC and buffered at pH=7.4 with Tris-hydroxymethyl aminomethane (TRIS) and HCl. This solution was used to coat the Na-Ti samples with OCP. These samples were immersed in this solution for 3 days producing a thick coating layer (Ti-OCP samples). The three surfaces (cp Ti, Na-Ti and octacalcium phosphate) were observed in a scanning electron microscope (SEM, JEOL, JSM 6460) coupled with energy-dispersive spectrometer (EDS).

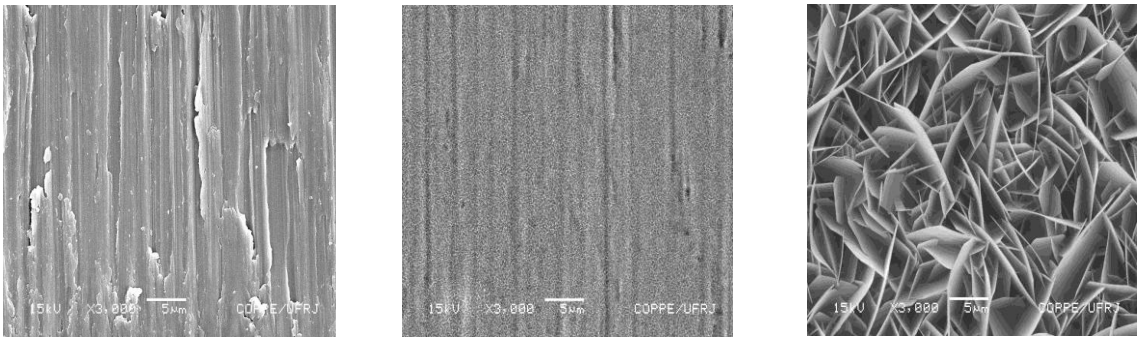
2.2 Cell Experiments

All the samples were sterilized with gamma radiation at 25 kGy. Cell-adhesion and spreading abilities were investigated by using pre-osteoblastic cells from Balb/c3T3 femur bone (FOST), differentiated in an appropriate medium. These cells were cultured in DMEM with 10% SFB (bovine fetal serum) and seeded ($3x10^4$ cells/well) on the three surface conditions for 24 hours. Polystyrene discs (Thermanox®) were used as control. At the end of the incubation period, the adherent cells were harvested and counted in a Neubauer chamber. In short, the cultures were rinsed three times with saline solution having neither sodium nor magnesium. Sodium saline buffer was used to remove non-adherent cells. Then cells were enzymatically detached from the disks chamber, fixed in formaldehyde and counted in Neubauer chamber. After three independent experiments, means and standard deviation values were submitted to variance analysis and Tukey post-test considering significant differences if $p<0.05$.

In addition, cell morphology was analyzed by scanning electron microscopy (SEM) after fixing attached cells with glutaraldehyde 2.5% in cacodilate buffer 0.1M plus calcium cloride 0.01 (pH 7.3) for 2 min and dehydration by hexamethyldisiloxane (HMDS).

3 RESULTS AND DISCUSSION

Figure 1 shows representative SEM images, with the same magnification of the studied surfaces: pure titanium (cp-Ti), Ti coated with sodium titanate (Na-Ti) and octacalcium phosphate coated titanium surfaces (Ti-OCP samples). These results indicated a clear difference on the three surfaces, concerning microstructure and chemical composition. OCP with the typical plate-like morphology was observed. The coating layer was free of cracks and its thickness was estimated in the range of 5-10 μm. An extensive surface characterization, including grazing-incidence X-ray diffraction and transmission electron microscopy was used to characterize OCP surface and can be found elsewhere [3].



(a) (b) (c)

Figure 1: Micrographs of surfaces: (a) cp Ti, (b) Na-Ti and (c) Ti-OCP.

More details of the Na-Ti microstructure can be observed in Figure 2 as well as its EDS spectrum. According to SEM micrographs, the Na-Ti layer showed a network microstructure with sub-micrometric

porosity due to NaOH attack. The typical elements of a sodium titanate layer (Na, Ti and O) were identified by EDS analysis.

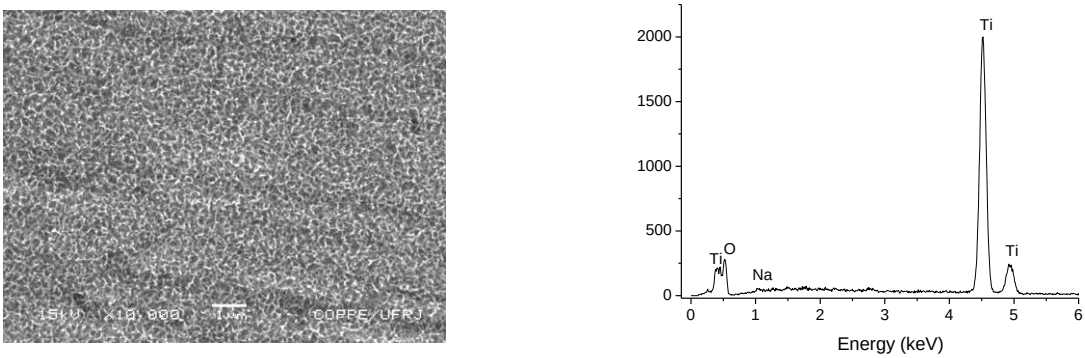


Figure 2: Micrographs and EDS spectrum of a Na-Ti layer.

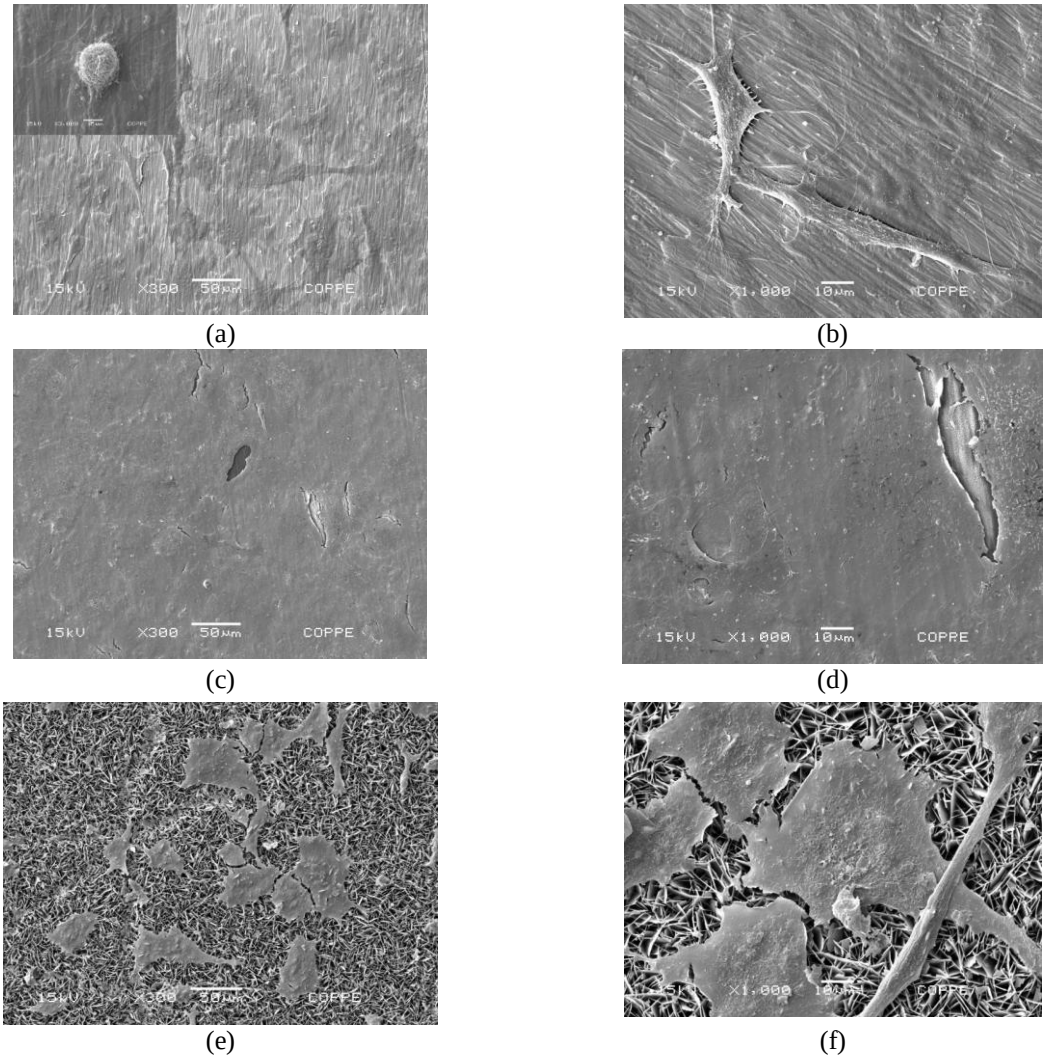


Figure 3: SEM micrographs of cells cultured for 24 hours on: (a-b) cp Ti, (c-d) sodium titanate (Na-Ti) and (e-f) Ti-OCP.

Both SEM and EDS analyses confirmed the chemical modification on Ti surface. Furthermore, sodium titanate is largely characterized, as it seems to promote an apatite layer formation after in vivo implantation, contributing to the binding between the tissue and the implant [14].

Cells morphology on the three surfaces is shown in Figure 3. Scanning electron microscopy revealed round shaped cells on cp Ti surface, although most of them showed random spreading, indicating that the sandpaper marks did not guide the cells' growth. On the other hand, round cells were not found on neither surface - Na-Ti and Ti-OCP - suggesting good spreading in comparison with the untreated titanium. Besides, a cell layer was observed on Na-Ti surface (Figure 3, c-d). A layer with cells can also be observed on OCP samples (Figure 3, e-f) but it does not cover all surfaces.

Cell attachment, spreading and subsequent proliferation are closely related to surface properties such as composition, roughness, wettability and morphology [10]. It was reported that cellular response on biomaterials involves the following steps: *serum proteins adsorption* on the substrate; contact of round cells on the substrate; cells attachment to the substrate and cells spreading on the substrate. In the attachment step, physico-chemical linkages are involved between cells and material surface by ionic and/or van der Walls forces [11].

The number of cells on the studied surfaces is an important parameter to determine if there is significant difference among the tested surfaces, Figure 4. The number of cells was significantly lower for Ti-OCP, Na-Ti and cp Ti when compared with control. Although the amount of cells was visually higher on titanate (Figure 3 c, d), it is interesting to notice that there was no statistical difference among the studied materials. Surprisingly, the CaP coating did not show a number of cells higher than on the other surfaces, although CaP coating on metallic surface is said to improve the contact of titanium with the bone. Similar trend was observed by SOCOL *et al*. [15], where Ti and OCP coating obtained by pulsed laser deposition were compared. Perhaps, the thick layer of OCP associated with plate-like morphology was not friendly to cell adhesion.

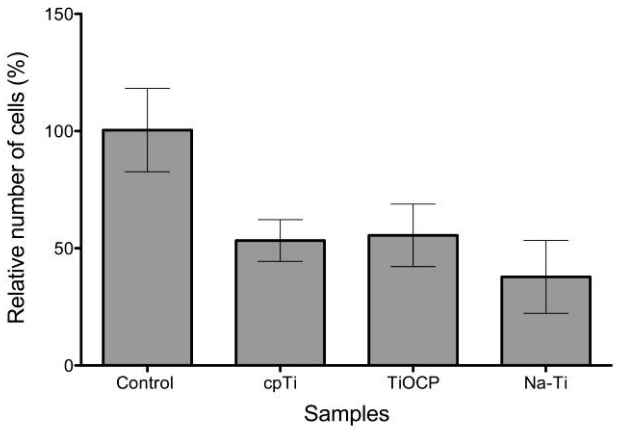


Figure 4: Relative number of adhered osteoblasts after 24 hours. Error bar indicates the confidence interval of 95% of mean value. Control = polystyrene.

Titanium and Ti alloys, either coated or uncoated with CaP, were studied by ZHENG *et al.* [16]. According to this work, the attachment and proliferation rate on the studied surfaces, up to 24 hours, are very similar. However, a different profile was observed at 72 and 144 hours, evidencing the positive effect of CaP coating in relation to another sample. Similar trend was observed in our work in a short cell culture period, 24 hours. The same behavior was observed by BIGI *et al.* [17], who studied Ti surfaces coated with OCP and Mn^{2+} doped carbonated HA. Despite three days' culture, there was no proliferation difference among the groups (Ti was used as control).

The outcomes of the present work, in accordance with the literature, emphasize the importance of performing cell viability studies for more than 24 hours. Therefore, a more detailed cell proliferation study is necessary and it should be carried out in order to understand all the steps involved in the studied surfaces cellular response.

4 CONCLUSION

All of the tested surfaces allowed cell adhesion and spreading, being, therefore, considered cytocompatible. Besides, titanium surface could be coated through biomimetic process; however, coating did

not increase cell adhesion in relation to cpTi and Na-Ti, in opposition to a higher number of cells on the polystyrene surface.

5 ACKNOWLEDGMENTS

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