Response *In Vitro* Human Cells to Hydroxyapatite Coatings on Titanium Substrates Synthesized by Sol-Gel Process

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**Abstract**

In biomaterials field, surface modifications of bio-inert implants allow optimization of their surface properties while retaining favorable bulk properties.

The objective of this study has been to improve the biological properties of commercially pure titanium grade 4, a material generally used for implant application. For this purpose, coatings, with composition similar to human mineralized bone hydroxyapatite, were developed by a sol-gel dip coating route and their biological performances were evaluated. Hydroxyapatite sol was synthesized via sol-gel using Ca(NO₃)₂·4H₂O and P₂O₅ as precursors. The sol was used to coat titanium substrates by means of dip-coating technique and the obtained films were heated on 600°C and 1000°C. The nature of the gel was investigated by X-Ray diffraction (XRD). Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy and scanning electron microscopy (SEM) were used for chemical and microstructural analysis of the coatings. Their potential application in the biomedical field was evaluated by bioactivity and biocompatibility tests. The samples were immersed in simulated body fluid (SBF) to evaluate bone-bonding ability. To investigate the effect of the interaction between the cells and the material, human mesenchymal stromal cells (hMSCs) were seeded onto the coated and uncoated substrates and the cells response was evaluated. Heat treated coatings show a microporous structure, ideal for cell adhesion. In fact, these samples appear to be more bioactive and biocompatible than untreated ones. Those results suggest that the coatings could have a bone-bonding capability *in vivo*.

**Keywords:** Biocompatibility; Dip Coating; Hydroxyapatite; Sol-Gel Synthesis; Titanium Grade 4 Substrates

**Introduction**

The aim of tissue engineering is to develop substitutes, organic or synthetic, which are able to restore or maintain the function of a damaged tissue within the human body. It represents a synergistic union between life sciences and engineering [1]. Tissue engineering should ideally produce tissue substitutes which can “grow” with the patient, overcoming the limitations of conventional treatments that are based on biomaterial implant and organ transplantation [2,3]. Scaffolds have to meet requirements such as biocompatibility, osseointegration ability, chemistry and topography of the surface able to promote adhesion, proliferation and differentiation of the cells, as well as controlled biodegradability [3,4]. Taking into account these fundamental characteristics, different types of materials were proposed to develop scaffolds specific for the tissue to be regenerated, such as natural, synthetic, semisynthetic and hybrid materials, also able to release biological molecules or to induce cellular differentiation [5-7]. Moreover, scaffolds for bone tissue engineering must possess adequate mechanical properties. For this reason, ceramic materials (i.e. hydroxyapatite, tricalcium phosphate, silica and zirconia) have been considered. These materials have proven to be biocompatible and bioactive, which are crucial features especially in the ortho-
In the current work, HA was synthesized using the sol-gel technique. Hydroxyapatite sol was used to coat a substrate made of titanium grade 4 (Ti-4), which is a material widely employed to design implants in the orthopedic, dental and orthodontic fields. The idea should be to associate the mechanical properties of titanium, suitable for the clinical use, with the excellent biological performance of HA. The dip coating technique was used to make thin layer on the Ti-4 substrates. To study the effect of the temperature on morphology and, thus, biological performance of the coatings, different heat-treatments was used to induce film densification and synthesis by-product removal. Scanning electron microscopy (SEM), attenuated total reflectance Fourier transform infrared (ATR-FTIR) and X-ray diffraction (XRD) analyses were performed to analyze the morphology and chemistry of the coating. Moreover, the evaluation of the bioactivity and bio-compatibility of the coated substrates was carried out and compared with those of the uncoated ones by bioactivity test and WST-8 assay respectively. Osteocalcin expression were investigated on human mesenchymal stromal cells (hMSCs) grow on samples for evaluation of osteogenic differentiation properties. All results were reported as a function of the temperature used for the heat treatment.

Materials and Methods

Sol-gel synthesis

Hydroxyapatite was synthesized using two different chemical reagents as precursors. Phosphoric pentoxide $(\text{P}_2\text{O}_5)$ $(0.006 \text{ mol})$ and calcium nitrate tetrahydrate $(\text{Ca(NO}_3)_2 \cdot 4\text{H}_2\text{O})$ $(0.02 \text{ mol})$ were dissolved in absolute ethanol $(\text{EtOH}, 99.8\%)$ to form $1\text{M}$ and $2.5\text{M}$ solutions, respectively. Therefore, after their mixing under stirring, a sol with a $\text{Ca/P}$ atomic molar ratio equal to $1.67$ (the same which was observed in biological HA) was obtained. Moreover, $\text{NH}_4\text{OH}$ solution was used to adjust the pH to $11$. All reagents and the solvent were produced by Sigma Aldrich. The synthesized sol was stirred at room temperature for $24\text{h}$ to obtain a clear solution. The flow chart of the synthesis process is shown in Figure 1.

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Commercially available pure Ti-4 disks (Sweden and Martina, Padua, Italy) of 8 mm in diameter were coated with hydroxyapatite sol by means of a KSV LM dip coater. Before coating, the substrates were soaked in acetone, cleaned in an ultrasonic bath and then subjected to a process of passivation in HNO_3 (65 wt%). The coating procedure was carried out 24h after synthesis, and a withdrawal speed equal to 15 mm/min was used. The coated substrates were heated on 600°C and 1000°C for 2h.

**Chemical and morphological analyses**

X-ray diffraction (XRD) analysis was carried out by a Philips diffractometer (Philips Electronic Instruments Co., Model PW 1730) to investigate the changes in material crystallinity as a function of the temperature. Therefore, powder of both untreated and heat-treated samples were scanned from 10° to 80° 2θ using CuKα radiation.

The ATR-FTIR analyses were also performed on both heat-treated and untreated HA samples using a Prestige-21 FTIR spectrometer equipped with an AIM-8800 infrared microscope (Shimazu, Japan) and an incorporated 3 mm diameter Ge ATR semicircular prism. Spectra were obtained in the 4000 - 650 cm⁻¹ range, using 45 scans at 8 cm⁻¹ resolution. The spectra were analysed by Prestige IRsolution software. Using a Quanta 200 FEI apparatus (Europe Company, Netherlands), scanning electron microscopy (SEM)/energy dispersive X-ray (EDX) spectroscopy was used to investigated the coating morphology. The samples were fixed on an aluminum stub with colloidal graphite and gold metalized using a K550X Sputter Coater (Emitech, UK).

**Bioactivity test**

The potential bone-bonding ability of a material, when implanted *in vivo*, can be predicted by the apatite-forming-ability test *in vitro*, according with Kokubo, *et al*. [21]. The test was carried out by soaking the titanium substrates, uncoated and coated, with HA after heating on 600°C and 1000°C, for 7, 14, and 21 days in a simulated body fluid (or SBF). That is a solution prepared by adding several salts in distilled water to have a concentration of ions similar to those of the human blood plasma. As the ratio between the total surface area exposed to the solution and its volume affects the formation of the HA layer it was kept equal to 10. A water bath was used to maintain the polystyrene bottles, where the samples in the solution were placed at 37°C. In order to avoid depletion of ionic species in the SBF solution, as a consequence of the biomineral nucleation, the solution was replaced every 2 days.

SEM/EDX analysis was used, after each soaking times, to evaluate the formation of an hydroxyapatite layer on the surface of the air dried samples.

**WST-8 assay**

To evaluate the coating biocompatibility, human mesenchymal stromal cells (hMSCs, Lonza) were seeded on coated and uncoated Ti-4 substrates. The evaluation of cell viability was carried out by the WST-8 Assay (Dojindo Molecular Technologies Inc., MD, USA), an indirect colorimetric method which allows to quantify the cell viability through the measure of the cellular metabolic activity.

hMSC cells were grown in DMEM medium (Gibco, CA, USA) enriched with 10 v/v% fetal bovine serum, 1% pen-strep, into a humidified incubator at 37°C, 5% CO_2 and 95% air. After amplification in a flask of 25 cm² and arrival in semi-confluence, a solution of trypsin (2.5 wt/v%) and EDTA (0.2 wt/v%) in PBS (Phosphate Buffered Saline), was used to detach the cells from the plate. 5∙10^3 cells were seeded on the surface of the coated and uncoated Ti-4 substrates. After 24 hours, the water-soluble purple-coloured WST-8 tetrazolium salt or [2 - (2-methoxy-4-nitrophenyl) -3 - (4-nitrophenyl) -5 - (2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was added to the culture medium. It penetrates the cellular membrane and is cleaved by mitochondrial dehydrogenases of the live cells, producing insoluble yellow-orange crystals of formazan. The quantification of the generated formazan can be carried out measuring the absorbance at 450 nm of the well-plates by UV-Vis spectrophotometer (Biomate 3, Thermo Scientific). This measure, thus, expresses the number of living cells.
Response In Vitro Human Cells to Hydroxyapatite Coatings on Titanium Substrates Synthesized by Sol-Gel Process

A low absorbance value means that the materials in contact with the cells is a cytotoxic agent, able to inhibit their mitochondrial activity. The reported values of absorbance were calculated as average of 3 determinations. The value recorded for cells grown on uncoated Ti-4 substrate was identified 100% of vitality.

Immunofluorescence tests

Human mesenchymal stromal cells were seeded on the coated disks as described previously and cultured for 7 days. Uncoated titanium disks were used as negative control. After 7 days, disks were washed in PBS and fixed with cold methanol (-20°C) for 5 min and permeabilized with a 0.1% Triton-X100 in PBS for 10 min. Subsequently cells were washed with PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 20 min. After a rinse in PBS, samples were incubated with mouse anti-osteocalcin antibody (Bio-Rad) diluted 1/200 in 1%BSA for 1h. After three washes in PBS, cells were incubated with alexa fluor 488 goat anti-mouse (ThermoFisher) diluted 1/500 in 1%BSA for 1h in dark room. For nuclei staining, samples were washed in PBS and incubated with To-Pro 3 iodide (Thermo-Fisher) diluted 1/1000 in 1%BSA for 1h in dark room. Stained cells were examined with Nikon Confocal Microscope C1 equipped with a EZ-C1 Software for data acquisition.

Results and Discussion

Results from XRD analysis of sol-gel coatings (a) untreated and treated (b) at 600°C and (c) at 1000°C are shown in Figure 2. The temperature used to sinter the materials plays a key role in the HA preparation. Accordingly, by increasing the sintering temperature the samples have shown several distinct and narrow peaks, thus suggesting an increase of the crystallite size.

![Figure 2: X-ray diffraction (XRD) of Hydroxyapatite sol-gel a) untreated b) treated at 600°C c) treated at 1000°C.](image)

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With regard to the diffractograms of the heat-treated samples, typical peak positions corresponding to reflections of HA, including those with higher intensity as well as the peak positions (2Φ) at 27.76, 31.80, 32.26, 33 and 53.06 which are related to the (002) (211), (112), (300), (004) reflections of HA [22]. These results demonstrated that at a temperature of 600°C were already formed crystalline phases in the HA coatings.

Figure 3 shows ATR-FTIR spectra of the hydroxyapatite coating (a) untreated, treated to (b) 600°C and (c) 1000°C.

In the spectra of untreated samples the bands at 3420 and 1650 cm⁻¹ are due to the water used in the synthesis. Moreover, strong bands at 1423, 1382 and 1354 cm⁻¹ ascribable to the asymmetric and symmetric stretching of nitrate ions [23,24] and sharp peaks at 1047 and 823 cm⁻¹ related to the bending vibrations of nitrate ions are visible. Indeed, only when the thermal degradation of nitrates occurs (at a temperature beyond 350°C) calcium ions enter into the network by diffusion. Before such temperature, calcium nitrate salt coats the material network and, thus, the FTIR nitrate signals appear strong.

However, the presence of the asymmetric and symmetric stretching of P-O bonds at 1090 and 960 cm⁻¹ proves that HA network was formed [25,26].

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After heating on 600°C, another peak related to the vibrational mode of hydroxyapatite P-O groups (1033 cm⁻¹) is evident (Figure 3b). Moreover, a sharp peak at 3570 cm⁻¹ appears due to -OH ions of hydroxyapatite. Finally, the presence of the typical peaks of CO₃²⁻ vibrations are visible at 1450 and 873 cm⁻¹ that prove the formation of carbonated hydroxyapatite.

HA spectrum of coating treated at 1000°C is reported in Figure 3c. The intensities of peaks at 1450 and 1075 cm⁻¹ are decreased since CO₃²⁻ is driven away as volatile gas at higher calcination temperatures. Moreover, the peak at 3570 cm⁻¹ disappears and a shift of peak related to P-O vibrational modes from 1033 cm⁻¹ to 1040 cm⁻¹ is visible, probably due to the increase of crystallinity recorded at this calcination temperature by XRD analysis.

Figure 4 shows SEM images of hydroxyapatite coatings heated on 600°C and 1000°C. No great differences were observed in both samples. They present a porous structure with micropores of 3 - 4 µm in diameter on the surface. This kind of structure is particularly favorable to cell access. EDX analysis shows that in the materials the Ca/P atomic ratio is 1.50, confirming that a carbonation process of hydroxyapatite occurred. It was caused by the reaction between the atmospheric CO₂ and the excess of calcium in the prepared samples.

After 7, 14 and 21 days of exposure to SBF, sol-gel hydroxyapatite coating (Figure 5) shows the globular formations which are typical of physiological hydroxyapatite. These are much more evident at 21 days. There were not significant differences in bioactivity between the two kinds of samples (600°C and 1000°C) and, for this reason, we reported on the overall progress of both samples. The Ca/P atomic ratio recorded on the globules by EDX was 1.30 for the presence of additional carbonates in the simulated body fluid solution.

**Figure 4:** SEM-EDS of hydroxyapatite coating after heat treatment at 600°C and 1000°C.

**Figure 5:** SEM-EDS of hydroxyapatite coating after soaking in SBF for 7, 14, 21 days.
Cell viability was reported as the percentage of UV absorbance recorded at 450 nm in the well-plate where there are the cells grown on HA coatings compared to cells grown on uncoated Ti-4, considered as 100% viable (Figure 6). The coated samples were more biocompatible than titanium and there were no significant differences between the two thermal treatments. These results may be also related to the synergic effect of the chemistry and surface topography of the coatings.

![Figure 6: Results of WST-8 assay expressed as % of viability of cells seeded on hydroxyapatite heat treated at 600°C and 1000°C](image)

Immunofluorescence test (Figure 7) show that cells grown on coated samples expressed osteocalcin (green fluorescence), while hMSCs grown on titanium have only the stained nuclei (blue fluorescence). This result suggests that the coating induces the production of osteocalcin, an important marker for the osteogenic differentiation.

![Figure 7: Immunofluorescence analysis of cells grow on: A) uncoated titanium; B) hydroxyapatite after 600°C; C) hydroxyapatite after 1000°C. In blue cells nuclei, green signal is osteocalcin.](image)
Conclusion

The coating of titanium substrates with HA obtained via sol-gel dip coating has been proved to be a suitable strategy to improve the biological performance of the substrates. XRD and FT-IR analyses show that heat-treatment (600°C and 1000°C) of the coatings induces the increase of crystallite size, the degradation of nitrates ions, which adversely affects the biological response to the material, and the carbonation of the HA. Moreover, SEM micrographs showed that the obtained coatings are microporous, regardless from temperature used in the heat-treatment. This surface morphology is suitable to promote cell adhesion and osseointegration. Results of the biological tests, indeed, showed that all coated samples are more bioactive and biocompatible of the uncoated ones. Also induces osteogenic differentiation in human mesenchymal stromal cells.

Bibliography

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