ECRONICON OPEN ACCESS

EC DENTAL SCIENCE
Research Article

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

Michelina Catauro*

Department of Industrial and Information Engineering, University of Campania "Luigi Vanvitelli", Via Roma 29, Aversa, Italy

***Corresponding Author:** Michelina Catauro, Department of Industrial and Information Engineering, University of Campania "Luigi Vanvitelli", Via Roma 29, Aversa (CE), Italy.

Received: April 27, 2017; **Published:** May 27, 2017

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 $Ca(NO_3)_2 \cdot 4H_2O$ and P_2O_5 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 orthope-

dic or dental fields [8,9]. In particular, the attention of several researcher groups has focused on hydroxyapatite (HA) as material to be used for producing artificial bones, scaffolds for tissue engineering and chromatographic packaging [10], due to its high bioactivity and adsorbability to several ions and organic molecules. Moreover, as a consequence of the chemical similarity with the mineralize phase of human bone, synthetic HA clearly shows a high affinity to host hard tissues. For this reason, HA is able to form chemical bonds with the host tissue. On the other hand, metallic implants interact weakly with the biological environment. In addition, metallic alloys are bioinert materials. Therefore, even though it does not induce a injurious reaction when implanted in the body, it does not integrate as a bioactive material would [11]. Therefore, the implant is isolated from the surrounding bone by the formation of a fibrotic capsule, causing the early failure of the implant [12]. In recent years, different coatings for metal alloys have been developed. Specifically, in the biomedical field these coatings have also been designed to improve the interaction with hard tissues [13,14]. Nanocrystalline HA has been synthesized by various processes such as hydrothermal [15], mechanochemical [16], precipitation [17], hydrolysis [18] and sol-gel methods [19]. If compared to other techniques, the sol-gel method is able to improve the HA chemical homogeneity as it involves a mixing of calcium and phosphorus precursors at the molecular level. Furthermore, this technique can form thin film coatings with a simple process such as dip coating. The dip coating technique allows complexly shaped substrates of different materials to be coated. Briefly, the device is immersed into the sol, prepared via sol-gel, and then withdrawn with a constant speed. Speed clearly represents an important parameter because it affects the thickness of the obtained layer [20].

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 biocompatibility 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 (P_2O_5) (0.006 mol) and calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$) (0.02 mol) were dissolved in absolute ethanol (EtOH, 99.8%) to form 1M and 2.5M solutions, respectively. Therefore, after their mixing under stirring, a sol with a Ca/P atomic molar ratio equal to 1.67 (the same which was observed in biological HA) was obtained. Moreover, NH_4OH 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 24h to obtain a clear solution. The flow chart of the synthesis process is shown in Figure 1.

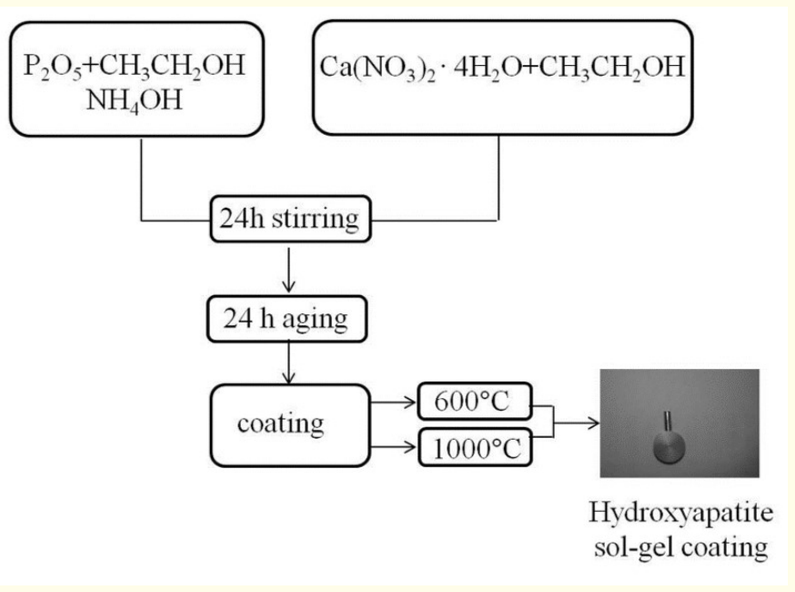

Figure 1: *Schematic flow process chart for the synthesis of HA.*

Coating procedure

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 a 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 heated-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 cm^2 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. $5 \cdot 10^3$ 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.

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 washes 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 (ThermoFisher) 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.

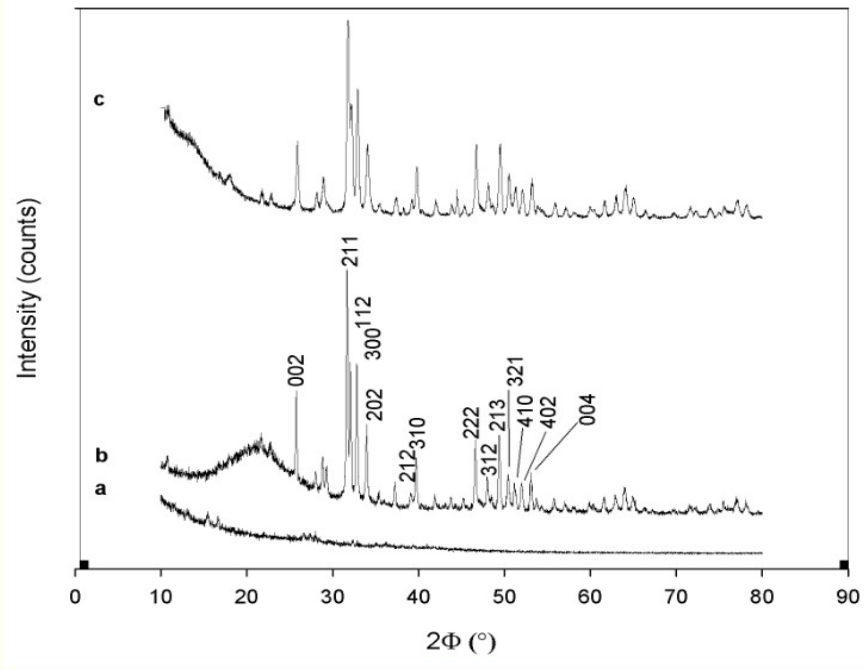

Figure 2: *X-ray diffraction (XRD) of Hydroxyapatite sol-gel a) untreated b) treated at 600°C c) treated at 1000°C.*

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.

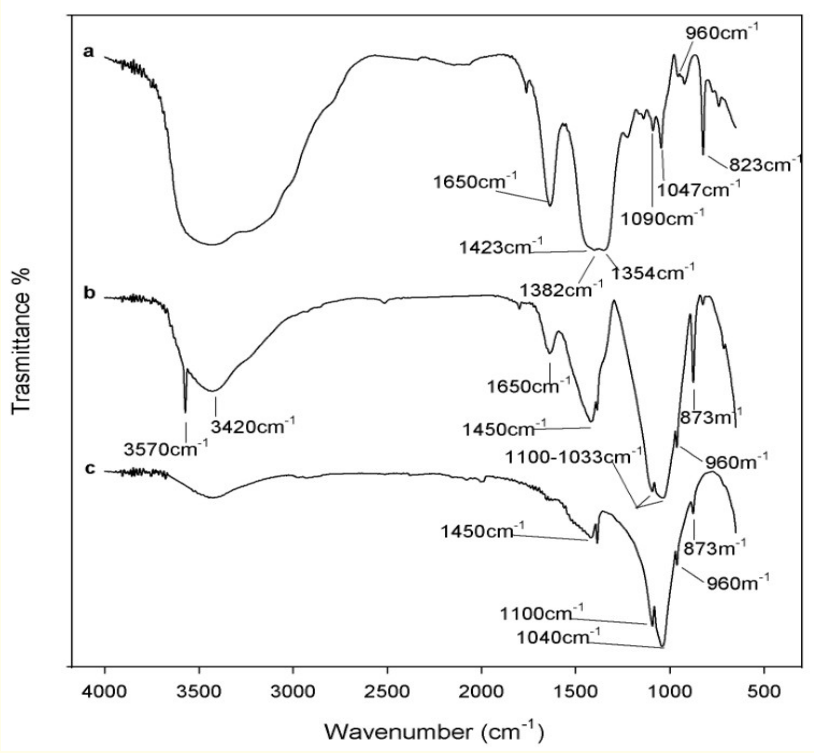

Figure 3: *ATR-FTIR spectra of hydroxyapatite layers after a) untreated, b) treated at 600°C c) treated at 1000°C.*

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].

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 CO_3^{2-} 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 875 cm^{-1} are decreased since CO_3^{2-} 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 accession. 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 CO_2 and the excess of calcium in the prepared samples.

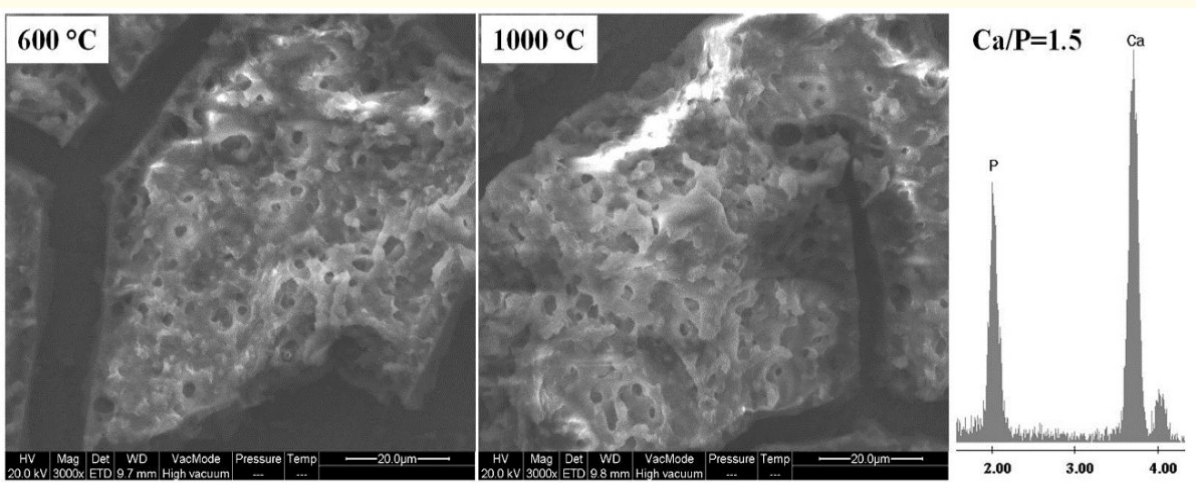

Figure 4: *SEM-EDS of hydroxyapatite coating after heat treatment at 600°C and 1000°C.*

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.

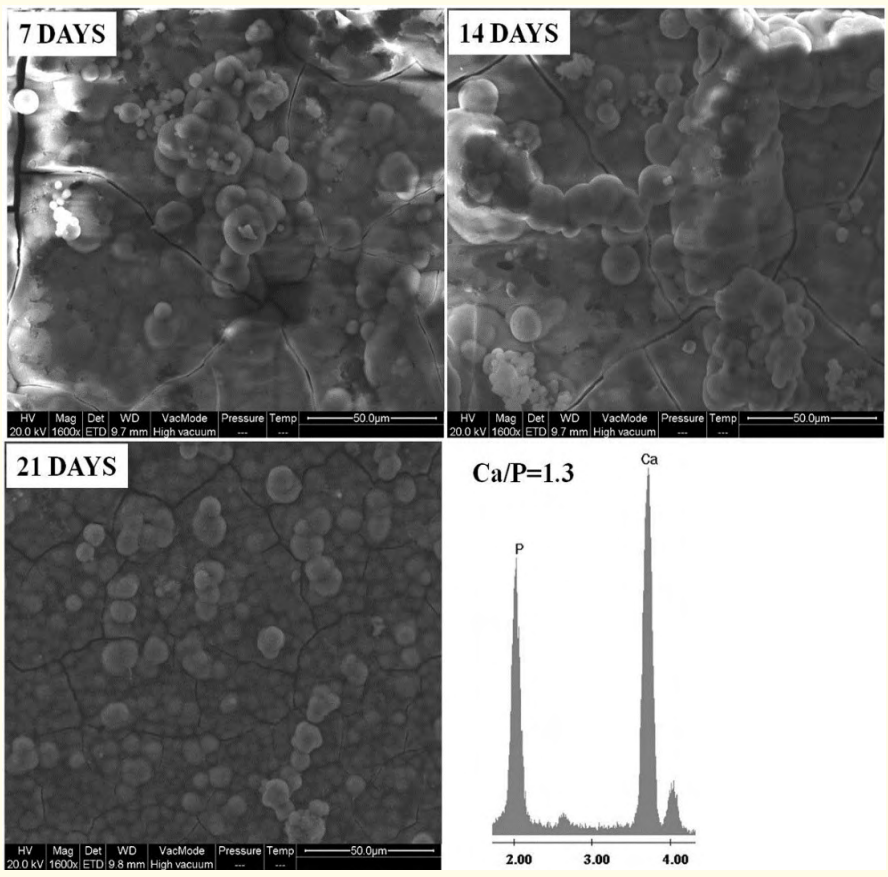

Figure 5: *SEM-EDS of hydroxyapatite coating after soaking in SBF for 7, 14, 21 days.*

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.

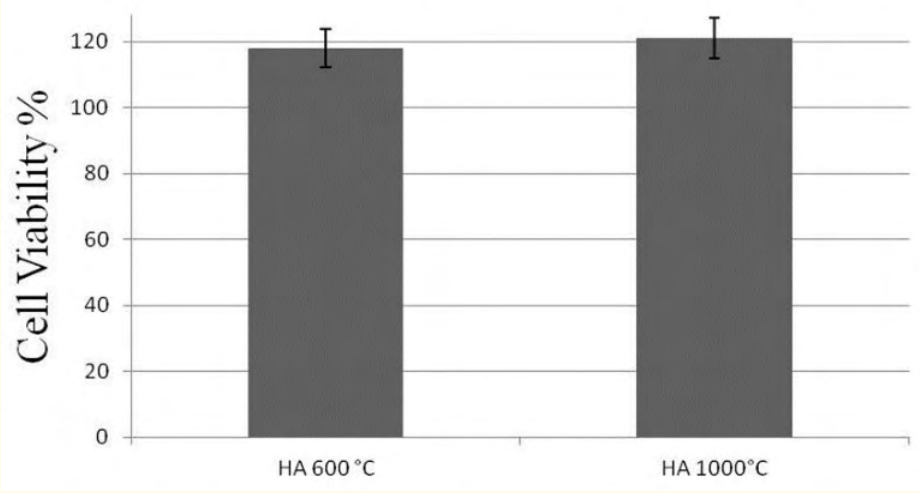

Figure 6: *Results of WST-8 assay expressed as % of viability of cells seeded on hydroxyapatite heat treated at 600°C and 1000°C*

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.

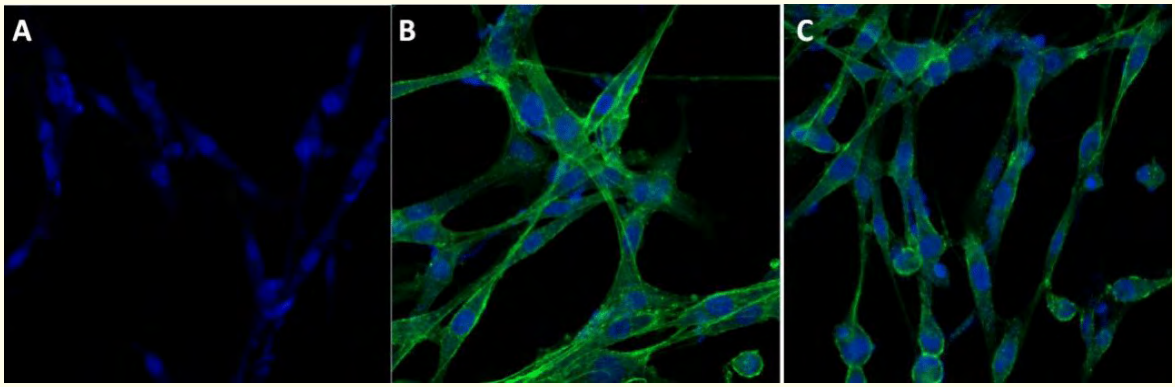

Figure 7: *Immunofluorescence analysis of cells grow on: A) uncoated titanium; B) hydroxyapatite after 600°C; C) hydroxyapatite after 1000°C. In blue cells nuclei, green signal is osteocalcin.*

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

1. Langer Robert. "Tissue Engineering". *Molecular Therapy* 1.1 (2000): 12-15.
2. Chapekar Mrunal S. "Tissue Engineering: Challenges and Opportunities". *Journal of Biomedical Materials Research* 53.6 (2000): 617-620.
3. Gloria A., *et al*. "Magnetic Poly(1-Caprolactone)/Irondoped Hydroxyapatite Nanocomposite Substrates for Advanced Bone Tissue Engineering". *Journal of the Royal Society Interface* 10.80 (2013): 20120833.
4. Cheung Hoi-Yan., *et al*. "A Critical Review on Polymer-Based Bio-Engineered Materials for Scaffold Development". *Composites, Part B, Engineering* 38.3 (2007): 291-300.
5. Catauro M., *et al*. "Sol-Gel Synthesis and Characterization of Sio2/Pcl Hybrid Materials Containing Quercetin as New Materials for Antioxidant Implants". *Materials Science and Engineering C* 58 (2016): 945-952.
6. Catauro M., *et al*. "Biological Response of Human Mesenchymal Stromal Cells to Titanium Grade 4 Implants Coated with Pcl/Zro2 Hybrid Materials Synthesized by Sol-Gel Route: In Vitro Evaluation". *Materials Science and Engineering C* 45 (2014): 395-401.
7. Catauro M., *et al*. "Tio2/Pcl Hybrid Materials Synthesized Via Sol-Gel Technique for Biomedical Applications". *Materials Science and Engineering C* 47 (2015): 135-141.
8. Catauro M., *et al*. "Coatings of Titanium Substrates with Xcao·(1 - X)Sio2 Sol-Gel Materials: Characterization, Bioactivity and Biocompatibility Evaluation". *Materials Science and Engineering C* 58 (2016): 846-851.
9. Catauro M., *et al*. "Sol-Gel Synthesis of Sio2-Cao-P2o5 Glasses: Influence of the Heat Treatment on Their Bioactivity and Biocompatibility". *Ceramics International* 41.10 (2015): 12578-12588.
10. Hench Larry L. "Bioceramics". *Journal of the American Ceramic Society* 81.7 (1998): 1705-1728.
11. Balazic Matej., *et al*. "Review: Titanium and Titanium Alloy Applications in Medicine". *International Journal of Nano and Biomaterials* 1.1 (2007): 3-34.
12. Jokstad Asbjorn., *et al*. "Quality of Dental Implants". *International Dental Journal* 53 (2003): 409-443.
13. Catauro M., *et al*. "Preparation of 0.7sio2·0.3cao/Pcl Hybrid Layers Via Sol–Gel Dip Coating for the Surface Modification of Titanium Implants: Characterization, Bioactivity and Biocompatibility Evaluation". *Journal of Sol-Gel Science and Technology* 76.2 (2015): 241-250.
14. Pourhashem S and A Afshar. "Double Layer Bioglass-Silica Coatings on 316l Stainless Steel by Sol-Gel Method". *Ceramics International* 40.1 (2014): 993-1000.

15. Yoshimura Masahiro., *et al*. "Hydrothermal Synthesis of Biocompatible Whiskers". *Journal of Materials Science* 29.13 (1994): 3399-3402.

16. Silva CC., *et al*. "Structural Properties of Hydroxyapatite Obtained by Mechanosynthesis". *Solid State Sciences* 5.4 (2003): 553-558.

17. Saeri MR., *et al*. "The Wet Precipitation Process of Hydroxyapatite". *Materials Letters* 57.24-25 (2003): 4064-4069.

18. Shih Wei-Jen., *et al*. "Crystal Growth and Morphology of the Nano-Sized Hydroxyapatite Powders Synthesized from Cahpo4·2h2o and Caco3 by Hydrolysis Method". *Journal of Crystal Growth* 270.1-2 (2004): 211-218.

19. Kim Il-Seok and Prashant N Kumta. "Sol-Gel Synthesis and Characterization of Nanostructured Hydroxyapatite Powder". *Materials Science and Engineering: B* 111.2-3 (2004): 232-236.

20. Klein LC. "Sol-Gel Technology for Thin Films, Fibers, Preforms, Electronics and Specialty Shapes". Park Ridge, NJ, USA: Noyes Publications (1988).

21. Kokubo Tadashi and Hiroaki Takadama. "How Useful Is Sbf in Predicting in vivo Bone Bioactivity?" *Biomaterials* 27.15 (2006): 2907-2915.

22. Venkateswarlu K., *et al*. "X-Ray Peak Broadening Studies of Nanocrystalline Hydroxyapatite by Williamson-Hall Analysis". *Physca B: Condensed Matter* 405.20 (2010): 4256-4261.

23. Irish Donald E and GE Walrafen. "Raman and Infrared Spectral Studies of Aqueous Calcium Nitrate Solutions". *Journal of Chemical Physics* 46.1 (1967): 378-384.

24. Piccirillo AM., *et al*. "Impulsive Wendroff's Type Inequalities and Their Applications". *AAPP Atti della Accademia Peloritana dei Pericolanti, Classe di Scienze Fisiche, Matematiche e Naturali* 90.2 (2012): A2.

25. Tiwari Babita., *et al*. "Preparation and Characterization of Phosphate Glasses Containing Titanium". *BARC Newsletter* 285 (2007): 167-173.

26. Piccirillo AM., *et al*. "Qualitative Analysis Behaviour of the Solutions of Impulsive Differential Systems". *AAPP Atti della Accademia Peloritana dei Pericolanti, Classe di Scienze Fisiche, Matematiche e Naturali* 89.2 (2011).

Volume 10 Issue 6 May 2017