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Abstract

The chemistry, structure and morphology of the implant surface have a great influence on the integration of an implant material with bone tissue. In this work, we applied Combinatorial Matrix-Assisted Pulsed Laser Evaporation (C-MAPLE) to deposit gradient thin films with variable compositions of Sr-substituted hydroxyapatite (SrHA) and Zn-substituted β-tricalcium phosphate (ZnTCP) on Titanium substrates. Five samples with different SrHA/ZnTCP composition ratios were fabricated by a single step laser procedure. SrHA was synthesized in aqueous medium, whereas ZnTCP was obtained by reaction at high temperature. Both powders were separately suspended in deionized water, frozen at liquid nitrogen temperature and used as targets for C-MAPLE experiments, which proceed via simultaneous laser vaporization of two distinct material targets. X-ray diffraction, scanning electron microscopy and energy dispersive X-ray spectroscopy analyses confirmed that the coatings contain the same crystalline phases as the as-prepared powder samples, with a homogeneous distribution of the two phosphates along deposited thin films. Human osteoclast precursor 2T-110 and human osteoblast-like cells MG63 were co-cultured on the coatings. The results indicate that osteoblast viability and production of osteocalcin were promoted by the presence of ZnTCP. On the other hand, SrHA inhibited osteoclastogenesis and osteoclast differentiation, as demonstrated by the observed increase of the osteoprotegerin/RANKL ratio and decrease of the number of TRAP-positive multinucleated cells when increasing SrHA amount in the coatings. The results indicate that the possibility to tailor the composition of the coatings provides materials able to modulate bone growth and bone resorption.

Keywords: strontium; hydroxyapatite; zinc; β-tricalcium phosphate; coatings; co-cultures

1. Introduction

Hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) are the most widely employed calcium phosphates (CaPs) for biomedical applications in the orthopedic field. HA is indeed the synthetic phase most similar to the inorganic component of bone, whereas the increasing interest toward β-TCP is due to its greater solubility and resorbability [1,2]. Ionic substitution can be utilized as a tool to improve the biological performance of CaPs and provide local delivery of ions with specific therapeutic properties [3]. The highly flexible structure of HA can accommodate a great variety of ions, both cations and anions, as testified by the elevated number of related publications [3-5]. However, ionic substitutions occur also in other CaPs, β-TCP included [6-10]. Among divalent ions, strontium has received increasing attention due to its recognized beneficial effect on bone metabolism, and to the introduction of strontium ranelate for the treatment of patients affected by osteoporosis [11,12]. Strontium can substitute for calcium up to 100 and 80 % in the structures of HA and β-TCP, respectively, whereas the percentage of substitution is much smaller in other CaPs [7,8,13,14]. Also zinc plays an important role on biomineralization. In fact, zinc deficiency has been reported to provoke reduction of bone density and risk of osteoporosis [15,16]. Similarly to what happens with other divalent cations with ionic radius smaller than calcium, just limited amounts of Zn can be generally incorporated into CaPs structures [10, 17-19]. In particular, the substitution for calcium occurs up to about 20 atom % both in HA and in β-TCP structures [8,17,20-22].

The next generation of implantable biomaterials should exhibit bioactive surfaces and interfaces able to modulate cellular behavior. The core concept in combinatorial materials science is based on the synthesis of compositional libraries of distinct compounds that preferably exert a synergistic influence in the composition-structure-properties relationship [23].We have recently shown that laser-based techniques are a promising alternative to the existing procedures for the micro- and nano-fabrication of combinatorial coatings in a single-step process [24-27]. Laser processing can control the morphology and/or chemistry features of biomaterials and the fabrication of hybrid compositional libraries. Combinatorial Matrix-Assisted Pulsed Laser Evaporation (C-MAPLE) was first introduced as an alternative to Pulsed Laser Deposition (PLD) for the synthesis of organic coatings, but proved beneficial for inorganic an hybrid layers as well. In the basic irradiation geometry of C-MAPLE, two cryogenic targets are synchronously evaporated by two pulsed laser beams [24,26]. Coatings with tailored properties could be thus obtained by independently controlling the key deposition parameters such as: laser fluence and repetition rate of the pulsed laser beams, the dynamic pressure inside the reaction chamber and the targets to substrate separation distance. The number of the applied laser pulses governs the thickness distribution within library; while the separation distance between the two focused beams influence the compositional spreading along gradient. Moreover, tuning doping concentrations in combinatorial coatings could be achieved by simply modifying their initial concentration in the frozen targets. The gradient of properties, which is reached by C-MAPLE, permits the accurate evaluation and selection of the best composition and morphology for one specific application in an one-step experiment. Furthermore, crystalline CaPs thin films can be grown at low substrate temperature [26].

In this study, we applied C-MAPLE to deposit gradient thin films with variable composition of Sr-substituted HA (SrHA) and Zn-substituted β-TCP (ZnTCP) on Titanium substrates. The aim of the work is to investigate how the compositional intermixing of two calcium phosphates (which differ in several aspects, including structure, morphology and solubility, and contain two different bioactive foreign ions) can be utilized to modulate bone cells response. To this purpose, we applied a model of co-culture of osteoblast and osteoclast in order to reproduce the *in vivo* microenvironment in which different cells interact with each other and with biomaterials.

2. Materials and methods

2.1 Synthesis and characterization of SrHA and ZnTCP crystals

SrHA nanocrystals were synthesized in N_2 atmosphere. 50 ml of solution with Sr/(Ca + Sr) ratio of 0.20 was prepared by dissolving 0.0432 mol of $Ca(NO_3)_2 \cdot 4\ H_2O$ and 0.0108 mol of $Sr(NO_3)_2$ in CO_2-free deionised water and adjusting pH to 10 with NH_4OH. The total concentration of $[Ca^{2+}] + [Sr^{2+}]$ was 1.08 M. 50 ml of 0.65 M $(NH_4)_2HPO_4$ solution, pH 10 adjusted with NH_4OH, was added drop-wise under stirring to the cationic solution heated at 90 °C. The precipitate was maintained in contact with the reaction solution for 5 h at 90 °C under stirring, then centrifuged at 10,000 rpm for 10 min and repeatedly washed with distilled water. The product was dried at 37 °C.

β-TCP was prepared by reaction of $CaCO_3$ and $CaHPO_4 \cdot 2H_2O$ powders in the molar ratio of 1:2, at 1000°C for 15 h. α-$Zn_3(PO_4)_2$ was prepared by reaction of $(ZnCO_3)_2[Zn(OH)_2]_3$ and $(NH_4)H_2PO_4$ in the molar ratio of 3:10, at 800°C for 12 h. In order to synthesize

ZnTCP with Zn/(Ca + Zn) ratio of 0.15, the appropriate mixture of β-TCP and α-$Zn_3(PO_4)_2$ was heat treated at 1000°C for 12 h.

Powder X-ray diffraction (XRD) patterns were recorded using a PANalytical X'Pert PRO powder diffractometer equipped with a fast X'Celerator detector. Ni-filtered CuKα radiation was used (λ = 0.154 nm, 40 mA, 40 kV). For phase identification the 2θ range was investigated from 10 to 60 2θ degrees with a step size of 0.1° and time/step of 100 s. The lattice parameters were determined by least-squares refinements from the well-determined positions of the most intense reflections using HighScore Plus software package (PANalytical). Silicon was used as internal standard.

Calcium, strontium and zinc contents in the solid products were determined by means of a Agilent 4200 microwave plasma- atomic emission spectrometer (MP-AES). Powders were previously dissolved in 0.1M HNO_3. Results from this analysis represent the mean value of three different measurements.

Morphological investigation of crystals was performed by transmission (TEM) and scanning electron microscopy (SEM). For TEM investigations, a small amount of powder was transferred onto holey carbon foils supported on conventional copper microgrids. A Philips CM 100 transmission electron microscope operating at 80 kV was used. For SEM investigations the samples were sputter-coated with Au before examination with a HITACHI S-2400 operating at 15 kV.

For infrared absorption analysis in attenuated total reflection (ATR) mode, powders were analyzed using a Bruker ALPHA FT-IR spectrometer equipped with a diamond unit, to collect 64 scans in the range 4000–400 cm^{-1} at a resolution of 4 cm^{-1}. Background correction and bands analysis were operated with OPUS software.

2.2 Laser synthesis and characterization of coatings

In C-MAPLE process, the beam of one laser was optically split into two beams, and then focused onto the surface of the targets, each one containing different frozen solutions [24,25]. Alternatively, different lasers could be considered, with different characteristics as pulse duration, wavelength, repetition rate, in order to match the absorption of the frozen solvent of each target. During multi-pulsed laser irradiation, the evaporated materials are assembled on the facing substrates. Consequently, a natural compositional gradient is generated along the longitudinal direction of the substrate due to substance fluxes intermixing (Figure 1).

The experimental protocol for combinatorial CaPs libraries synthesis was similar to those described in Refs. [24-26]. Briefly, 0.2 g of each nano-crystalline powder, SrHA and ZnTCP, were homogenously suspended in 20 ml distilled water by ultrasonical stirring. Then, 3 to 5 ml of each solution were poured into a two concentric rings holder. Accordingly, the approach was conceived to avoid unwanted mixing. The holder was immersed in liquid nitrogen for 15 min and solutions were frozen. They were used as solid targets in reaction chamber where a cooler supplied with liquid nitrogen flow preserve them frozen during multi-pulse laser irradiation and evaporation. In a single-step process, the synchronized evaporated materials were collected onto 5 distinct Ti substrates of 12 mm diameter (substrate temperature 150°C; target-substrate distance 5 cm). We applied 20,000 laser pulses (laser fluence 1.1 J cm^{-2}; dynamic pressure $2 \cdot 10^{-2}$ mbar) to grow ~400 nm thin films. The optimal experimental conditions for C-MAPLE transfer and immobilization of SrHA and ZnTCP were carefully adjusted to preserve materials characteristics (*e.g.* stoichiometry, crystallinity and functionality).

The samples were labeled A, B, C, D and E, where the composition varies from 100% SrHA (A) to 100% ZnTCP (E).

XRD measurements on the coatings were performed using a PANalytical X'Pert PRO powder diffractometer equipped with a fast X'Celerator detector. Ni-filtered CuKα radiation was used (λ = 0.154 nm, 40 mA, 40 kV). The 2θ range was investigated from 24 to 34° (2θ) with a step size of 0.067° and time/step of 3000 s.

Morphological investigations of thin films were performed using a HITACHI S-2400 scanning electron microscope operating at 15 kV. The samples were sputter coated with gold before examination. Energy dispersive X-ray spectrometry (EDS) analyses were performed on uncoated specimens.

For atomic force microscopy (AFM) imaging a Veeco Nanoscope 3D instrument was used. The samples were analyzed in tapping mode using a E scanner (maximum scan size 15 μm) and phosphorus (n) doped silicon probes (spring constant 20–80 N/m; resonance frequency 250–290 kHz; nominal tip radius<10 nm). Roughness parameters, namely arithmetic mean roughness (Ra), root-square roughness (Rq), and the vertical distance between the highest and lowest points within the evaluation length (Rt), were recorded.

Films adherence to Ti substrate was measured by "pull-out" method. The investigation was carried out with a standardized DFD Instruments PAThandy adhesion tester AT101 (maximum force=1 kN), equipped with stainless steel testing elements (dollies with a diameter of Φ=2.8 mm) that were glued to films surface with a cyano-acrylate one-component epoxy adhesive, type E1100S. After gluing and cleaning, the samples were placed in a stabilized oven (Venticell) for thermal curing at 130°C for 1h. The detachment of the dollies was achieved by the gradual increase of the pull-out force by means of a

hydraulic head until fracture occurred. The procedure was carried out according to ASTM D4541 and ISO 4624 standards. The tests were performed in triplicate.

2.3. In vitro tests

2.3.1 In vitro co-culture model

Cell experiments were carried out on coatings deposited on Ti substrates and sterilized by gamma-rays (Cobalt-60) at a dose of 25kGy. Human osteoclast precursor 2T-110 (OC, Poietics™ Osteoclast Precursor Cell System, Lonza Walkersville, Inc., MD, USA) and human osteoblast-like cells MG63 (OB, Istituto Zooprofilattico Sperimentale IZSBS, Brescia, Italy) were used for the co-culture model.

Pre-osteoclasts were plated at a concentration of $3x10^4$ cells/well in the bottom of 24-wells plates e cultured in DMEM additioned with macrophage colony-stimulating factor (MCSF, 25ng/ml) and receptor activator for κB factor ligand (RANKL, 30ng/ml) in standard condition, at 37°C±0.5 with 95% humidity and 5% CO_2±0.2 to activate cell differentiation.

OB were previously expanded in DMEM supplemented with 10% FCS, 1% antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), β-glycerophosphate (10^{-4}M) and ascorbic acid (50 µg/ml), counted, seeded at a concentration of $2x10^4$ cells on material samples at different gradient of SrHA and ZnTCP (from A to E). Then material samples with OB were co-cultured in the same wells with OC. Medium was a mixture of each cell type medium according to cell density proportion. Control cultures (CTR) were performed on cells plated on culture plates.

2.3.2 Cells viability and morphology

Proliferation and viability of co-cultured OB and OC was separately evaluated on disassembled co-cultures, transferring samples with OB in empty wells, by WST1

colorimetric reagent test (Roche Diagnostics GmbH, Manheim, Germany) at the end of experimental time. The assay is based on the reduction of tetrazolium salt to a soluble formazan salt by a reductase of the mitochondrial respiratory chain, active only in viable cells. 100 µl of WST1 solution and 900 µl of medium (final dilution: 1:10) were added to wells containing OC or OB, and the multi-well plates were incubated at 37°C for the next 4 h. Supernatants were quantified spectrophotometrically at 450 nm with a reference wavelength of 625 nm. Results of WST1 are reported as optical density (OD) and directly correlated with the cell number.

At 7 days OB viability and morphology were observed by the Live/Dead® assay (Molecular Probes, Eugene, OR, USA), according to the manufacturer's instructions. Samples were visualized using an inverted microscope equipped with an epifluorescence setup (Eclipse TiU, NIKON Europe BV, NITAL SpA, Milan, Italy): excitation/emission setting of 488/530 nm to detect green fluorescence (live cells) and 530/580 nm to detect red fluorescence (dead cells).

For SEM investigation, osteoblasts grown on the materials were fixed in 2.5% glutaraldehyde, in phosphate buffer 0.01 M (pH 7.4) for 1 h, and then dehydrated in a graded ethanol series. After a passage in hexa-methyldisilazane, the samples were air-dried and sputter-coated with Pd. SEM investigation was carried out using a Hitachi S-2400 instrument operating at 15 kV.

TRAP staining was performed, after 7 days of co-culture, to assess osteoclast morphology and differentiation starting form mononucleated cells, according to manufacturer's instructions (SIGMA, Buchs, Switzerland). The positive cells developed red colour of different intensity. Osteoclastogenesis was evaluated by counting the number of TRAP-positive multinucleated cells (three or more nuclei each cell), under the microscope by a

semiautomatic software (NIS-Elements AR 4.30.01). Results are given as percentage of OC control culture considered as 100%.

2.3.3 Immunoenzymatic assays

At the end of experimental time, after 7 days of culture, the supernatant was collected from all wells and centrifuged to remove particulates, if any. Aliquots of supernatant were dispensed in Eppendorf tubes for storage at -70°C and assayed with the following immunoenzymatic kits: Alkaline Phosphatase (ALP, Cloud-Clone Corp., Wuhan, China), Osteocalcin (OSTC, e-Bioscience, Bender MedSystems, Vienna, A), Osteoprotegerin (OPG, Boster Biological Technology, Ca, USA), Receptor Activator for Nuclear factor KB Ligand (RANKL, Boster). Data are normalized by WST1 values.

2.3.4 Statistical Analysis

Statistical evaluation of data was performed using the software package SPSS/PC^{+} Statistics ^{TM} 23.0 (SPSS Inc., Chicago, IL USA). The results presented are the mean of six independent values. Data are reported as mean ± standard deviations (SD) at a significance level of $p<0.05$. After having verified normal distribution and homogeneity of variance, a one-way ANOVA was done for comparison between groups. Finally, a post hoc multiple comparison test was performed to detect significant differences among groups.

3. Results and discussion

3.1 Characterization of SrHA and ZnTCP crystals

The XRD pattern of the product of the synthesis of hydroxyapatite in the presence of strontium (SrHA) is characterized by a number of peaks which indicate the presence of hydroxyapatite as unique crystalline phase (Figure 2). However, the position of the

diffraction peaks are slightly shifted towards smaller angles in comparison with reference HA, in agreement with an enlargement of the interplanar spacings and, as a consequence, of the unit cell. The evaluation of the cell parameters gives values of a = 9.464(3) Å and c = 6.923(3) Å, which are larger than those obtained for pure HA (a = 9.421(1) Å and c = 6.876(1) Å) and suggest that the relatively big Sr^{2+} is incorporated into the lattice structure. The enlargement of the unit cell is congruous with Strontium content, which amounts to about 18 atom %, as determined by MP-AES, and in agreement with previous results [13].

TEM images of SrHA show small needle- or plate-like crystals, with mean length appreciably shorter than those of HA (Figure 3). The presence of strontium into HA lattice influences also the infrared absorption spectrum. Most of the absorption bands in the ATR-FTIR spectrum of SrHA appear slightly broadened in comparison with those characteristic of pure hydroxyapatite (HA). In particular, the OH^- libration band, which falls at 630 cm^{-1} in the spectrum of HA [28], is not appreciable. Moreover, the phosphate symmetric stretching and bending modes are slightly shifted at lower wave numbers in comparison to pure HA [13, 28] (Figure S1, Table S1).

The powder X-ray diffraction pattern of ZnTCP and β-TCP are reported in Figure 2. The synthesized ZnTCP sample does not exhibit any extra peak when compared to β-TCP, demonstrating that it is constituted of a single crystalline phase. The lattice parameters of ZnTCP evaluated from the XRD pattern are a = 10.34(1) Å and c =37.34(8) Å, which are slightly smaller than those obtained for pure β-TCP (a = 10.43(1) Å and c = 37.38(6) Å) in agreement with a partial substitution of Zn^{2+} for Ca^{2+} in the β-TCP structure. In fact, Zn^{2+} displays a smaller ionic radius (0.74 Å) than Ca^{2+} (0.99 Å), and its incorporation

into β-TCP lattice has been previously reported to provoke a decrease of the volume of the unit cell [8,17,29]. Zn content of ZnTCP is 15 atom %, close to the value previously reported as the maximum amount the structure can support [17]. Although the replacement of calcium with zinc into β-TCP does not significantly affect the sharpness of the XRD peaks (Figure 2), its influence on the ATR-FTIR spectrum is remarkable and suggests a reduction of crystallinity. In fact, the absorption bands of ZnTCP are broader and less resolved than those of β-TCP, as shown in Figure S1. Moreover, several bands appear shifted at lower wave numbers in comparison with those of β-TCP (Table S1). The presence of Zn displays a significant effect also on the morphology of the material, which is not constituted of the characteristic particles with round edges as β-TCP, but of much bigger irregular aggregates with sharp edges, as shown in Figure 3.

3.2 Structural and morphological characterization of the coatings

Typical X-ray diffraction patterns of the thin films deposited by C-MAPLE are given in Figure 4. Although the resolution, as well as the relative intensity, of the peaks is lower than in the XRD patterns of the respective powder samples, it is evident that there is no phase variation during the deposition of the films.

SEM images (Figure 5) show that the surface of A thin film is characterized by a granular morphology, with grain dimensions of the order of tens of nanometers. At variance, the surface of E displays the presence of bigger aggregates. A part of these aggregates shows irregular shape and sharp edges, whereas others exhibit the characteristic round edges as β-TCP, which most likely has been recovered during the process of plasma evaporation and deposition. Both small grains and bigger aggregates can be distinguished on the

surface of the thin films containing both SrHA and ZnTCP (from B to D), as shown in Figure 5 for C. EDS analysis and maps reported in Table S2 and Figure 5 confirm the homogeneous distribution of Sr and Zn ions in the different samples.

In agreement with the different morphology of SrHA and ZnTCP thin films, the roughness parameters, Ra, Rq and Rt, evaluated by AFM analysis (Figure S2) increase slightly on going from A to E. Average values measured for A were: Ra = 0.454 ± 0.058 µm, Rq = 0.583 ± 0.072µm, Rt = 3.471 ± 0.127µm, whereas the values of the other samples increased up to Ra = 0.669 ± 0.075 µm, Rq = 0.829 ± 0.095 µm, Rt = 4.861 ± 0.182 µm, measured for E.

The adherence to substrate of the thin films was explored by pull-out investigation. In all cases, the adhesion values are between 16 to 18 MPa, i.e. superior to the threshold of 15 MPa requested by ISO13779-2:2008 standard for implantology coatings.

3.3 In vitro study

MG63 and 2T110 were chosen to conduct the in vitro test as well-characterized cells, exhibiting the most important markers of differentiation and a standard behavior.

Osteoblast and osteoclast viability and differentiation were measured after 1 week of co-culture of cells with SrHA and ZnTCP deposited in different relative amounts on Ti disks using combinatorial-MAPLE (from A to E samples), and CTR. Results of the present study demonstrated a trend related to the different compositions of the thin films for both OB and OC.

In detail, OB grown on SrHA (A) samples showed viability similar to CTR. At variance, the increase of the presence of ZnTCP provoked a significant increase of the values of viability in comparison with both A and CTR (B,C: $p<0.05$), which reached the highest

levels in D and E ($p<0.05$ versus CTR and $p<0.005$ versus A) (Figure 6a). Data suggest that ZnTCP significantly improves osteoblast proliferation, both alone and in presence of different amounts of SrHA. SrHA did not show cytotoxic effects and did not significantly affect OB proliferation. Figure 6b shows representative images of Live & Dead staining of OB grown on samples at 7 days. Cells are well adherent onto the surface of all different materials. Very few red colored cells were detected, demonstrating that the differences in cells number were probably due to different rate of cell proliferation and that no apoptotic or necrotic events occurred.

In agreement, osteoblasts on the surface of the coatings appear well attached and spread, and display a number of filopodia, as it can be appreciated in the SEM images reported in Figure 7. Summarizing, besides the differences found between groups, the proliferation of OBs was never lower than CTR and cells showed a regular growth and no signs of toxicity.

Cells viability of OC is showed in Figure 8. Viability values decreased from E, which was similar to CTR, to the lowest value found for A ($p<0.005$ versus CTR and $p<0.0005$ versus E), displaying to a dose-effect trend. ZnTCP seems also to have an effect on OC number and differentiation, but the differences between ZnTCP (E) and CTR samples did not reached statistical significance. The presence of SrHA affected not only OC number but also OC differentiation, as shown in Figure 8.

The evaluation of differentiated OC through the number of TRAP-positive multinucleated cells, demonstrated that OC number and differentiation decreased significantly on increasing the presence of SrHA in the thin films. Data were also statistically correlated (Pearson test 0.962, $p<0.005$). Examples of TRAP staining performed at 7 days of co-culture are shown in Figure S3.

To assess if OB maintained or modified their activity on the different samples in comparison to CTR, two common markers of osteoblastic activity were chosen and evaluated after 7 days of co-culture: ALP and OSTC, respectively as early and late marker of OB differentiation. ALP activity of OB cultured on all samples was slightly higher than CTR, even if the values did not reach statistical significance (Figure 9).

OSTC values of all samples containing ZnTCP, both alone (E vs CTR, $p<0.005$) and in combination with SrHA (B, C, D vs CTR, $p<0.05$) were significantly higher when compared to CTR, which did not differ from A. Results in the present experimental conditions showed that both SrHA and ZnTCP did not alter ALP activity of OB, not affecting the early phases of OB differentiation. On the contrary, ZnTCP strongly improved the differentiated state of OB and the mineralization process, as indicated by the enhanced production of OSTC (Figure 9).

OB and OC cultured together influence each other and modulate the balance between bone deposition and bone resorption. In order to evaluate the effects of SrHA and ZnTCP and their combinations on the co-culture in relation to this aspect, OPG and RANKL were measured at the end of experimental time, as they and their ratio play a role in OC differentiation and activity. Results showed that SrHA stimulated OPG production, reaching statistical significance in B group ($p<0.05$), and reducing RANKL in A, B and C with respect to CTR ($p<0.05$) (Figure S4). The OPG/RANKL ratio showed a highly significant difference between the samples with higher concentrations of SrHA (A, B, C) and both E and CTR (Figure 9).

It is known that Sr is effective in counteracting bone resorption and its local action when incorporated into HA has been already demonstrated by a number of in vitro and in vivo studies [8,12,30-36]. Zn is involved in bone metabolism, with an action primarily aimed

at stimulating OB activity [15,16]. Furthermore, it was previously shown that biomimetic Zn-TCP can induce and stimulate faster osteogenic differentiation of mesenchymal stem cells to osteoblasts than pure β-TCP [37]. On the contrary, β-TCP containing small amounts of substituted/doped Zn has been reported to exhibit an inhibitory effect on osteoclast number and activity in vitro [19,38].

Chemical and morphological analyses of the samples after incubation in cell medium for 7 days (in the absence of cells) show that the thin films still completely cover the titanium substrates (Figure S5). The morphology of the coatings surface appears modified when compared to the as-prepared samples, most likely due to a partial dissolution of the deposited phosphates. Furthermore, the presence of some round shaped aggregates could be ascribed to deposition from the biological medium. However, the results of EDS analysis indicate that the ionic composition is roughly maintained (Table S2). The results of the present study demonstrated that both SrHA and ZnTCP had effects on OB and OC co-culture. In particular, SrHA decreased OC number and the process of differentiation, as showed by WST1, TRAP and the pathway related to OPG/RANKL, in agreement with previous data [32-34]. On the other hand, ZnTCP enhanced OB proliferation and promote OB differentiation as stated by WST1 and OSTC values. Although both OC viability and TRAP levels on E (ZnTCP) displayed smaller values than on CTRL, the differences did not reached statistical significance. As a consequence, the results of in vitro tests vary as a function of composition of the thin films.

4. Conclusion

Combinatorial-MAPLE technique has been successfully applied to deposit blended thin films with a gradient composition of Sr-substituted hydroxyapatite and Zn-substituted β-TCP on Titanium substrates. The response of osteoblast and osteoclast co-cultured on the coatings is modulated by the graded composition and varies with the relative content of SrHA and ZnTCP. In particular, the data indicate that the presence of SrHA inhibits osteoclast viability and differentiation, whereas ZnTCP displays a beneficial action on the mineralization process promoting osteoblast proliferation and osteocalcin production. The intermediate compositions, containing both SrHA and ZnTCP, couple the positive effects on osteoblast with the inhibitory action on osteoclast and provide materials with tailored capability, via laser processing parameters, in order to enhance and accelerate bone repair.

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Abbreviations

C-MAPLE	Combinatorial Matrix-Assisted Pulsed Laser Evaporation
HA	hydroxyapatite
β-TCP	β-tricalcium phosphate
SrHA	Sr-substituted hydroxyapatite
ZnTCP	Zn-substituted β-tricalcium phosphate
CaPs	Calcium phosphates
AFM	Atomic Force Microscopy
SEM	Scanning electron microscopy
XRD	X-ray diffraction
EDS	Energy dispersive X-ray spectrometry
OB	Osteoblast
OC	Osteoclast
DMEM	Dulbecco's modified Eagle's medium
WST1	Tetrazolium salt
ALP	Alkaline phosphatase
OSTC	Osteocalcin
OPG	Osteoprotegerin
RANKL	Receptor Activator for Nuclear factor KB Ligand
TRAP	Tartrate-resistant acid phosphatase

References

[1] S. Bose , S. Tarafder, Calcium phosphate ceramic systems in growth factor and drug delivery for bone tissue engineering: A review, Acta Biomater. 8 (2012) 1401–1421.

[2] W. Habraken, P. Habibovic, M. Epple, M. Bohner, Calcium phosphates in biomedical applications: materials for the future? Mater. Today 19 (2016) 69-87.

[3] A. Bigi, E. Boanini, M. Gazzano, Ion substitution in biological and synthetic apatites. In: Aparicio C, Ginebra MP eds, Biomineralization and Biomaterials, C. Foundamentals and applications, 1st Edition, Sawston, Cambridge (UK): Woodhead Publishing (imprint Elsevier), (2015) pp. 235-266

[4] J.H. Shepherd, D.V. Shepherd, S.M. Best, Substituted hydroxyapatites for bone repair, J. Mater. Sci. Mater. Med. 23 (2012) 2335–2347.

[5] A. Haider, S. Haider, S. Soo Han, I.K. Kang, Recent advances in the synthesis, functionalization and biomedical applications of hydroxyapatite: a review, RSC Adv. 7 (2017) 7442-7458.

[6] E. Boanini, M. Gazzano, A. Bigi, Ionic substitutions in calcium phosphates synthesized at low temperature, Acta Biomater. 6 (2010) 1882–1894.

[7] E. Boanini, M. Gazzano, K. Rubini, A. Bigi, Collapsed octacalcium phosphate stabilized by ionic substitutions, Cryst. Growth Des. 10 (2010) 3612–3617.

[8] K. Matsunaga, T. Kubota, K. Toyoura, A. Nakamura, First-principles calculations of divalent substitution of Ca^{2+} in tricalcium phosphates, Acta Biomater. 23 (2015) 329–337.

[9] M. Frasnelli, V.M. Sglav, Effect of Mg^{2+} doping on beta–alpha phase transition in tricalcium phosphate (TCP) bioceramics, Acta Biomater. 33 (2016) 283–289.

[10] M. Zhang, C. Wu, H. Li, J. Yuen, J. Chang, Y. Xiao, Preparation, characterization and in vitro angiogenic capacity of cobalt substituted β-tricalcium phosphate ceramics, J. Mater. Chem. 22 (2012) 21686–21694.

[11] S.J. Gallacher, T. Dixon, Impact of treatments for postmenopausal osteoporosis (bisphosphonates, parathyroid hormone, strontium ranelate and denosumab) on bone quality: a systematic review, Calcif. Tissue Int. 87 (2010) 469–484.

[12] P.J. Marie, Strontium ranelate: a novel mode of action optimizing bone formation and resorption, Osteoporos. Int. 16 (2005) S7–S10.

[13] A. Bigi, E. Boanini, C. Capuccini, M. Gazzano, Strontium-substituted hydroxyapatite nanocrystals, Inorg. Chim. Acta 360 (2007) 1009–1016.

[14] S.J. Saint-Jean, C.L. Camiré, P. Nevsten, S. Hansen, M.P. Ginebra, Study of the reactivity and in vitro bioactivity of Sr-substituted α-TCP cements, J. Mater. Sci. Mater. Med. 16 (2005) 993–1001.

[15] R.J.M. Lynch, Zinc in the mouth, its interactions with dental enamel and possible effects on caries; a review of the literature, Int. Dent. J. 61 (Suppl. 3) (2011) 46–54.

[16] A. Gür, L. Çolpan, K. Nas, R. Çevik, J. Saraç, F. Erdoğan, M.Z. Düz, The role of trace minerals in the pathogenesis of postmenopausal osteoporosis and a new effect of calcitonin, J. Bone Miner. Metab. 20 (2002) 39–43.

[17] A. Bigi, E. Foresti, M. Gandolfi, M. Gazzano, N. Roveri, Isomorphous substitutions in β-tricalcium phosphate: the different effects of zinc and strontium, J. Inorg. Biochem. 66 (1997) 259–265.

[18] D.V. Shepherd, K. Kauppinen, R.A. Brooks, S.M. Best, An in vitro study into the effect of zinc substituted hydroxyapatite on osteoclast number and activity, J. Biomed. Mater. Res. Part A 102 (2014) 4136–4141.

[19] Y. Yamada, A. Ito, H. Kojima, M. Sakane , S. Miyakawa, T. Uemura, R.Z. LeGeros, Inhibitory effect of Zn2+ in zinc-containing beta-tricalcium phosphate on resorbing activity of mature osteoclasts, J. Biomed. Mater. Res. Part A 84 (2008) 344–352.

[20] A. Bigi, E. Foresti, M. Gandolfi, M. Gazzano, N. Roveri, Inhibiting effect of zinc on hydroxylapatite crystallization, J. Inorg. Biochem. 58 (1995) 49-58

[21] M. Li, X. Xiao, R. Liu, C. Chen, L. Huang, Structural characterization of zinc-substituted hydroxyapatite prepared by hydrothermal method, J. Mater. Sci.: Mater. Med. 19 (2008) 797–803.

[22] F. Ren, R. Xin, X. Ge, Y. Leng, Characterization and structural analysis of zinc-substituted hydroxyapatites, Acta Biomater. 5 (2009) 3141–3149.

[23] X.D. Xiang, I. Takeuchi, Combinatorial Materials Synthesis, ISBN: 0-8247-4119-6, Marcel Dekker, Inc., New York, 2003.

[24] E. Axente, F. Sima, L.E. Sima, M. Erginer, M.S. Eroglu, N. Serban, C. Ristoscu, S.M. Petrescu, E.T. Oner, I.N. Mihailescu, Combinatorial MAPLE gradient thin film assemblies signalling to human osteoblasts, Biofabrication 6 (2014) 035010

[25] F. Sima, E. Axente, I. Iordache, C. Luculescu, O. Gallet, K. Anselme, I.N. Mihailescu, Combinatorial Matrix Assisted Pulsed Laser Evaporation of a biodegradable polymer and fibronectin for protein immobilization and controlled release, Appl. Surf. Sci. 306 (2014) 75–79.

[26] E. Boanini, P. Torricelli, F. Sima, E. Axente, M. Fini, I. N. Mihailescu, A. Bigi, Strontium and zoledronate hydroxyapatites graded composite coatings for bone prostheses, J. Colloid Interface Sci. 448 (2015) 1–7.

[27] F. Sima, E. Axente, C. Ristoscu, O. Gallet, K. Anselme, I. Mihailescu, Bioresponsive surfaces and interfaces fabricated by innovative laser approaches, Adv. Mater. Interfaces (2016) 427-462.

[28] B.O. Fowler, Infrared studies of apatites. II. Preparation of normal and isotopically substituted calcium, strontium, and barium hydroxyapatites and spectra-structure-composition correlations, Inorg. Chem. 13 (1974) 207-214.

[29] S. Gomes, J.M. Nedelec, E. Jallot, D. Sheptyakov, G. Renaudin, Unexpected mechanism of Zn^{2+} insertion in calcium phosphate bioceramics, Chem. Mater. 23 (2011) 3072–3085.

[30] E. Bonnelye, A. Chabadel, F. Saltel, P. Jurdic, Dual effect of strontium ranelate: stimulation of osteoblast differentiation and inhibition of osteoclast formation and resorption in vitro, Bone 42 (2008) 129–138.

[31] N. Neves, D. Linhares, G. Costa, C. C. Ribeiro, M. A. Barbosa, *In vivo* and clinical application of strontium-enriched biomaterials for bone regeneration: A systematic review, *Bone Joint Res.* 6 (2017) 366–375.

[32] C. Capuccini, P. Torricelli, E. Boanini, M. Gazzano, R. Giardino, A. Bigi, Interaction of Sr-doped hydroxyapatite nanocrystals with osteoclast and osteoblast-like cells, J. Biomed. Mater. Res. Part A 89 (2009) 594–600.

[33] C. Capuccini, P. Torricelli, F. Sima, E. Boanini, C. Ristoscu, B. Bracci, G. Socol, M.Fini, I.N. Mihailescu, A. Bigi, Strontium-substituted hydroxyapatite coatings

synthesized by pulsed-laser deposition: in vitro osteoblast and osteoclast response, Acta Biomater. 4 (2008) 1885–1893.

[34] E. Boanini, P. Torricelli, M. Fini, A. Bigi, Osteopenic bone cell response to strontium-substituted hydroxyapatite, J. Mater. Sci. Mater. Med. 22 (2011) 2079–2088.

[35] Y.F. Li, X.P. Shui, L. Zhang, J. Hu, Cancellous bone healing around strontium-doped hydroxyapatite in osteoporotic rats previously treated with zoledronic acid, J. Biomed. Mater. Res. Part B: Appl. Biomater. 104 (2016) 476–481.

[36] F. Salamanna, G. Giavaresi, A. Parrilli, P. Torricelli, E. Boanini, A. Bigi, M. Fini, Antiresorptive properties of strontium substituted and alendronate functionalized hydroxyapatite nanocrystals in an ovariectomized rat spinal arthrodesis model. Mater. Sci. Eng. C–Mater. Biol. Appl. (2017) in press doi: 10.1016/j.msec.2017.11.016

[37] J. Chou, J. Hao, H. Hatoyama, B. Ben-Nissan, B. Milthorpe, M. Otsuka, Effect of biomimetic zinc-containing tricalcium phosphate (Zn–TCP) on the growth and osteogenic differentiation of mesenchymal stem cells, J. Tissue Eng. Regen. Med. 9 (2015) 852–858.

[38] M. Roy, G.A. Fielding, A. Bandyopadhyay, S. Bose, Effects of zinc and strontium substitution in tricalcium phosphate on osteoclast differentiation and resorption. Biomater. Sci. 1 (2013) 74–82.

Captions to the figures.

Figure 1. Scheme of the C-MAPLE process. Fabrication of five samples in a single step process (labeled A, B, C, D and E), where the composition varies from 100% SrHA (A) to 100% ZnTCP (E).

Figure 2. XRD of SrHA and ZnTCP powders, compared with those of HA and β-TCP.

Figure 3. TEM images of HA and SrHA, and SEM images of β-TCP and ZnTCP.

Figure 4. Powder X-ray diffraction patterns of the A (SrHA), and E (ZnTCP) thin films.

Figure 5. SEM images and EDS maps of A, C and E thin films as-prepared. In the maps Green: Sr; Blue: Zn.

Figure 6. (a) Osteoblast (OB) viability after 7 days of co-culture on the different samples. Cells cultured on culture plates represent control (CTR). Statistical analysis is reported in the figure (*$p<0.05$, **$p<0.005$).

OB: * B, C vs A, CTR; * D, E vs CTR; ** D, E vs A;

(b) Live & Dead fluorescence staining of OB grown onto experimental samples with different composition and CTR. In all samples cells displayed regular morphology, no sign of suffering or apoptosis. Images were in agreement with viability test (WST1) (optical microscope, 10x magnification).

Figure 7. SEM images of osteoblast grown on A, C and E thin films at 7 days.

Figure 8. Osteoclast (OC) viability and activity after 7 days of co-culture on the thin films. Cells cultured on culture plates represent control (CTR). Statistical analysis is reported in the figure (*$p<0.05$, **$p<0.005$, ***$p<0.0005$).

OC: * A vs B ** A vs C and CTR;*** A and C vs E; ** B vs D and E; *** B vs CTR; ** C and D vs CTR; * D vs E;

TRAP %: percentage of cells positive to staining were counted for each group, considering CTR as 100%. *A, B, C, D vs E, CTR.

Figure 9. Alkaline Phosphatase, Osteocalcin and OPG/RANKL ratio, as markers of OB differentiation were evaluated in cell supernatant after 1 week of OB-OC co-culture on material samples and CTR. Statistical analysis is reported in the figure (*$p<0.05$, **$p<0.005$, ***$p<0.0005$).

ALP: no differences among groups;

OSTC: * B, C, D vs CTR; * E vs C; ** E vs CTR.

OPG/RANKL ratio: ** A, C vs E, CTR; *** B vs E, CTR