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Gradient coatings of strontium hydroxyapatite/zinc β-tricalcium phosphate as a tool to modulate osteoblast/osteoclast response

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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.

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

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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 a 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 dropwise 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 an Agilent 4200 microwave plasma- atomic emission spectrometer (MP-AES). Powders were previously dissolved in 0.1 M 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 (Fig. 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

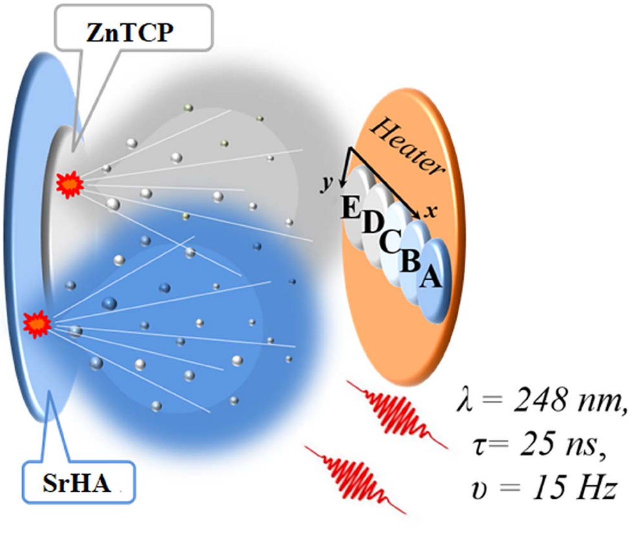

Fig. 1. Scheme of the C-MAPLE process. Fabrication of five samples in a single step process (labeled A, B, C, D and E), where the composition varies from 100% SrHA (A) to 100% ZnTCP (E).