Journal of Colloid and Interface Science 448 (2015) 1–7

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

Strontium and zoledronate hydroxyapatites graded composite coatings for bone prostheses

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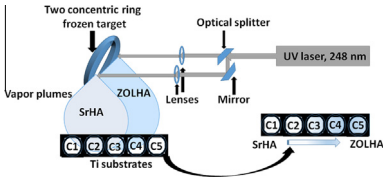
GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 November 2014
Accepted 31 January 2015
Available online 9 February 2015

Keywords:
Combinatorial-MAPLE
Coatings
Strontium
Zoledronate
Hydroxyapatite
Co-cultures

ABSTRACT

Both strontium and zoledronate (ZOL) are known to be useful for the treatment of bone diseases associated to the loss of bone substance. In this work, we applied an innovative technique, Combinatorial Matrix-Assisted Pulsed Laser Evaporation (C-MAPLE), to deposit gradient thin films with variable compositions of Sr-substituted hydroxyapatite (SrHA) and ZOL modified hydroxyapatite (ZOLHA) on Titanium substrates. Compositional gradients were obtained by simultaneous laser vaporization of the two distinct material targets. The coatings display good crystallinity and granular morphology, which do not vary with composition. Osteoblast-like MG63 cells and human osteoclasts were co-cultured on the thin films up to 21 days. The results show that Sr counteracts the negative effect of relatively high concentration of ZOL on osteoblast viability, whereas both Sr and ZOL enhance extracellular matrix deposition. In particular, ZOL promotes type I collagen production, whereas Sr increases the production of alkaline phosphatase. Moreover, ZOL exerts a greater effect than Sr on osteoprotegerin/RANKL ratio and, as a consequence, on the reduction of osteoclast proliferation and activity. The deposition method allows to modulate the composition of the thin films and hence the promotion of bone growth and the inhibition of bone resorption.

1. Introduction

Orthopaedic implants require materials with reliable strength, toughness and resistance to wear and corrosion [1,2]. These mechanical requirements are well fulfilled by metallic materials, which however are not able to form a stable bond to bone tissue. Osteointegration can be improved by coating the metal surface with a thin film of calcium phosphate [3]. Functionalization of calcium phosphates with bioactive substances can significantly enhance the performance of the implant. In particular, the positive effect of bisphosphonates (BPs) has been demonstrated on both healthy and osteoporotic animals [4]. As a matter of fact, BPs strongly inhibit bone degradation [5–7], which justifies their widespread employment for the treatment of disorders of bone

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http://dx.doi.org/10.1016/j.jcis.2015.01.088
0021-9797/

metabolism involving loss of bone substance [8]. Functionalization of calcium phosphate coatings with BPs has been generally achieved by soaking the implant in a BP solution [4]. We have recently shown that it is possible to use Matrix-Assisted Pulsed Laser Evaporation (MAPLE) to synthesize BP-hydroxyapatite (BP-HA), namely alendronate–hydroxyapatite composite coatings [9]. Thanks to the presence of BP, these thin films inhibit osteoclast proliferation and differentiation, whereas they promote osteoblast growth, viability and early differentiation [9]. A similar effect on inhibition of osteoclast activity and promotion of osteoblast differentiation has been reported for strontium via *in vivo* and *in vitro* studies [10,11]. Moreover, the influence of Sr on bone cells is maintained when it is incorporated into HA structure [12–14]. The co-presence of Sr and a potent amino bisphosphonate, zoledronate, in HA nanocrystals has been shown to exhibit a combined, dose dependent, effect on osteoblast promotion and osteoclast inhibition [15].

Recent improvements of MAPLE technique for the biochemical functionalization of surfaces in the field of biomaterials engineering rely upon the development of new concepts for the synthesis of novel materials. The Combinatorial-MAPLE (C-MAPLE) technique is a new approach for the fabrication of gradient organic or inorganic thin films, in view of obtaining bioactive artificial surfaces able to modulate and control cells behavior [16]. Distinct areas of a polymeric binary gradient have been shown to modulate the osteoblast extracellular signal-regulated kinase signaling with different propensity [17]. There are still plenty of challenges to be considered for the third-generation of biomaterials [18]. The goal of this study was the engineering of cell/biomaterial surface interface in order to control cell behavior, with major implications for tissue repair or scaffolds [19].

Due to materials complexity, the conventional "step by step" synthesis of biomaterials with different fixed composition and their subsequent analyses are both time consuming and expensive. With the present study, we propose C-MAPLE as an innovative synthesis method of gradient thin films with variable composition, *in situ* and in a single-step process. The aim is to evaluate the compositional intermixing of Sr-substituted hydroxyapatite (SrHA) and zoledronate modified hydroxyapatite (ZOLHA) for tailoring osteoblast promotion and osteoclast inhibition responses. The fabrication of an array of different chemical and physical features allows cells to be exposed to combinatorial nanostructures to be screened for a synergistic behavior.

2. Materials and methods

2.1. Synthesis and characterization of SrHA and ZOLHA nanocrystals

SrHA nanocrystals were synthesized in N_2 atmosphere using 50 ml of solution with Sr/(Ca + Sr) ratio of 0.1, prepared by dissolving the appropriate amount of $Ca(NO_3)_2{\cdot}4\,H_2O$ and $Sr(NO_3)_2$ in CO_2-free deionized water and adjusting pH to 10 with NH_4OH. The total concentration of $[Ca^{2+}] + [Sr^{2+}]$ was 1.08 M. The solution was heated at 90 °C and 50 ml of 0.65 M $(NH_4)_2HPO_4$ solution, pH 10 adjusted with NH_4OH, was added drop-wise under stirring. 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 overnight.

ZOLHA was obtained following the above procedure in the absence of Sr and by adding disodium zoledronate tetrahydrate (Chemos GmbH) to the phosphate solution. Concentration of zoledronate was 14 mM, calculated on final volume.

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 Cu Kα 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. Data used for cell parameters calculations were collected counting for 1200 s at each 0.033° (2θ), and then processed with the Rietveld routine of the HighScore Plus software package (PANalytical).

Calcium and strontium contents in the solid products were monitored by means of an ICP spectrometer (ICP Optima 4200DV, Perkin Elmer). Powders were previously dissolved in 0.1 M HCl. Results from this analysis represent the mean value of three different determinations.

Bisphosphonate content was determined spectrophotometrically via complex formation with Fe(III) ions using a Varian Cary50Bio instrument (λ = 290 nm) [20].

For Transmission Electron Microscopy (TEM) investigations, a small amount of powder was dispersed in ethanol and submitted to ultrasonication. A drop of the suspension 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 Fourier Transform-Infrared Spectroscopy (FT-IR) absorption analysis, 1 mg of the powdered sample was carefully mixed with KBr (250 mg, infrared grade) and pelletized under a pressure of 10 tons for 2 min. The pellets were analyzed using a Nicolet 380 FT-IR spectrophotometer to collect 32 scans in the range 4000–400 cm^{-1} with a resolution of 4 cm^{-1}.

2.2. Synthesis and characterization of SrHA and ZOLHA coatings

The experiments of thin coatings deposition were carried out in a vacuum reaction chamber [16,17]. 0.2 g of each powder, SrHA and ZOLHA respectively, were homogenously suspended separately in 20 ml distilled water by ultrasonically stirring. Three ml of each solution were poured into a two concentric ring holder. This way, any possible mixing was avoided. The holder was immersed in liquid nitrogen for 15 min and the solutions were frozen. They were further used as solid targets in the reaction chamber where a cooler supplied with liquid nitrogen flow kept them frozen during multi-pulse laser irradiation and vaporization. We have used a KrF* excimer laser source (λ = 248 nm, τ_{FWHM} = 25 ns) operating at a repetition rate of 3 Hz. The beam was split into two beams, directed by mirrors and focalized with lenses onto the surface of the two concentrically targets, SrHA and ZOLHA respectively (Fig. 1).

In a single experimental run, the synchronized evaporated materials were collected onto 5 distinct Ti substrates of 12 mm diameter. 20,000 laser pulses were applied to grow 0.4 μm thin films, which is sufficient for the *in vitro* tests under considerations. The samples were labelled C-1, C-2, C-3, C-4 and C-5, where the composition varies from C-1 (SrHA) to C-5 (ZOLHA). The set-up allows placing the standard reference compositions (SrHA and

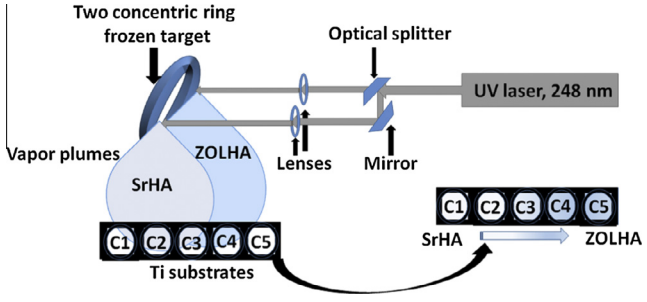

Fig. 1. Schematic of the C-MAPLE set-up.