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Antiresorptive and anti-angiogenetic octacalcium phosphate functionalized with bisphosphonates: An in vitro tri-culture study

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ABSTRACT

Development of new materials for the local administration of bisphosphonates (BPs) is aimed to avoid the negative side effects of prolonged systemic use of these potent drugs. In this work, we synthesized octacalcium phosphate (OCP) in the presence of two potent BPs and obtained a single crystalline phase up to a zoledronate and alendronate content of 3.5 wt% and 5.2 wt%, respectively. Both BPs provoke minor structural modifications and a reduction of the crystal dimensions of OCP, which suggests a preferential interaction of the BPs with the structure of the calcium phosphate. Alendronate containing samples display increased values of zeta potential with respect to that of OCP, and an initial burst release of the BP in solution. At variance, the zeta potential of zoledronate functionalized samples decreases on increasing the content of zoledronate, which is not appreciably released in solution. Bone microenvironment response to the composite materials was investigated in vitro using a triculture model. BP functionalized samples downregulate the viability of the cells, sustain osteoblast differentiation and accelerate the production of collagen type I and osteocalcin. At variance, they inhibit monocyte differentiation into osteoclast and provoke a dose dependent reduction of VEGF production, exhibiting antiresorptive and anti-angiogenetic properties that can be usefully exploited for the local treatment of abnormal bone losses.

Statement of Significance

Bisphosphonates (BPs) are powerful drugs for the treatment of bone diseases. However, BPs systemic administration suffers several undesirable side effects, which stimulate the development of suitable systems for their local administration. In this study we functionalized octacalcium phosphate (OCP) with alendronate and zoledronate in order to get biomaterials able to couple the good biological performance of OCP with the therapeutic properties of the BPs. The results provide novel information on the interaction between these two potent BPs and octacalcium phosphate. Moreover, the triculture in vitro study indicates that the synthesized composite materials stimulate the production of bone extracellular matrix, inhibit monocytes differentiation into osteoclasts and downregulate the release of Vascular Endothelial Growth Factor (VEGF) in a dose dependent way. The data allow to state that the new composite materials can be usefully employed for the local treatment of diseases involving abnormally high bone resorption.

1. Introduction

Bisphosphonates (BPs) are routinely employed for the treatment of high-turnover bone diseases related to an imbalance between osteoblast bone formation and excessive osteoclast bone resorption, such as osteoporosis, Paget's disease, hypercalcemia and metastatic cancer [1]. Furthermore, the results of a number of pre-clinical studies indicate that BPs display also antitumor activities [2,3]. In particular, amino-bisphosphonates (N-BPs) such as alendronate (AL) and zoledronate (ZOL) inhibit farnesyl pyrophosphate synthase activity in the mevalonate pathway, hindering the prelynation of small GTPases signaling proteins. This mechanism blocks many osteoclast activities and it is suggested to be responsible for the direct antitumor effect of N-BPs [2,3]. Moreover, indirect anticancer activities of N-BPs include interaction with macrophages and endothelial cells [4]. In fact, both ZOL and AL have been reported to inhibit endothelial cell adhesion,

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migration and proliferation, and to interfere with tumor angiogenesis [5–8]. However, N-BPs potentiality as effective anticancer drugs is remarkably affected by their unfavorable pharmacokinetics, since most of systemic administrated N-BPs rapidly binds to the skeleton or is cleared via renal filtration [9]. Poor bioavailability is just one of the drawbacks of the systemic administration of these drugs, which have several side effects, including osteonecrosis of the jaw and atypical subtrochanteric fractures [10,11]. Local administration at specific bone sites has been proposed as an alternative strategy to deliver N-BPs and avoid the potentially negative effects of their prolonged systemic use [12]. Calcium phosphates powders, cements and coatings are among the main delivery systems which have been proposed to this aim, whereas hydroxyapatite and β-tricalcium phosphate are the most used CaPs in this field [13–19]. In particular, we previously showed that hydroxyapatite (HA) can be functionalized with alendronate, as well as with zoledronate, through synthesis in aqueous solution [19,20]. BPs incorporation into HA nanocrystals, which reached a maximum amount of about 7 wt% both in HA-AL and in HA-ZOL, yielded materials able to exert a beneficial influence on in vitro osteoblast proliferation and differentiation, and to inhibit osteoclast proliferation and activity. In the present work, we explored the possibility to functionalize octacalcium phosphate (OCP) with alendronate and zoledronate, with the aim to get new biomaterials for the local treatment of diseases characterized by abnormal bone loss. OCP has been proposed as a valid alternative to HA for the preparation of biomaterials for hard tissue repair, due to its high speed of resorption and osteoconductivity, which has been related to its rapid conversion into apatite in a biological environment [21–24]. In fact, OCP is considered the precursor phase of biological apatites, and its triclinic crystal structure exhibits remarkable similarities with the hexagonal HA [25,26]. The possibility to functionalize OCP with BPs through synthesis in aqueous solution, in order to get materials able to couple the good biological performance of OCP with the therapeutic properties of the bisphosphonates, has not been explored up to now. Herein we carried out a chemical, structural and morphological characterization of the products obtained by synthesizing OCP in the presence of increasing concentrations of AL and ZOL. Bone cells response to the synthesized materials was tested in vitro using a triculture model involving osteoblasts, osteoclast and endothelial cells in order to mimic bone microenvironment. The functionalized materials sustain osteoblast activity, whereas they inhibit endothelial cells and osteoclast differentiation, displaying anti-resorptive and anti-angiogenetic properties, which could be usefully exploited for the local treatment of abnormal bone losses.

2. Materials and methods

2.1. Synthesis and characterization

2.1.1. Synthesis

The synthesis of OCP was carried out by dropwise addition of 250 ml of 0.04 M $Ca(CH_3COO)_2$ (over a period of 50 min) into 750 ml of a phosphate solution containing 5 mmol of Na_2HPO_4 and 5 mmol of NaH_2PO_4 at starting pH 5. The reaction was carried out at 70 °C under mechanical stirring. After 30 min the precipitate was filtered, repeatedly washed with distilled water and dried at 37 °C.

Similarly the synthesis of OCP in the presence of Alendronate was carried out by dropwise addition of 250 ml $Ca(CH_3COO)_2$ solution into 700 ml of a phosphate solution containing 5 mmol of Na_2HPO_4 and 5 mmol of NaH_2PO_4 at starting pH 5; afterwards the resulting slurry was stored under stirring for 30 min and then 50 ml of the sodium alendronate trihydrate (Chemos GmbH) solution was added dropwise over a period of 10 min. Immediately after the addition of Alendronate, the precipitate was filtered, repeatedly washed with distilled water and dried at 37 °C. Alendronate concentration was in the range 0.1–1.5 mM, calculated on the final volume. As a consequence samples were labeled as OALXX where XX indicates the concentration of BP in solution (expressed as mM).

The synthesis of OCP in the presence of Zoledronate was performed in the same condition of the modified synthesis of OCP-Alendronate. Disodium zoledronate tetrahydrate (Chemos GmbH) concentration was in the range 0.1–1.5 mM, calculated on the final volume. Samples were labeled as OZOLXX similarly to OALXX ones.

2.1.2. X-ray diffraction analysis

X-ray diffraction analysis was carried out by means of a PANalyticalX'Pert PRO powder diffractometer equipped with a fast X'Celerator detector. Ni-filtered CuKa radiation was used (40 mA, 40 kV). For phase identification the 2θ range was investigated from 3 to 60 2θ degrees with a step size of 0.1° and time/step of 100 s. To evaluate the coherence lengths of crystalline domains and to perform the full profile pattern refinement, further X-ray powder data were collected with a fixed counting time of 400 s for each 0.033/step. Silicon was used as internal standard. The coherence lengths of crystalline domains were calculated according to the Scherrer formula [19]. For cell parameters evaluation, data were processed with the Rietveld routine of the HighScore Plus software package (PANalytical).

XRD analysis at increasing temperature was performed by using an Anton Paar TTK-450 sample stage. The temperature was increased at 20 °C/min and the data collection was performed at the temperatures reported on the figures by scanning from 3.5 to 40° 2θ degrees counting 40 s each 0.05° step (with the fast X'Celerator detector an XRD scan was collected in 40 s).

2.1.3. Morphological analysis

Morphological investigations of crystals were performed using a Phenom ProX desktop-scanning electron microscope at beam acceleration voltage of 10 kV. The samples were observed as prepared and not sputter coated before examination. The ImageJ® picture analyzer software was used to estimate the mean crystals dimensions, averaging the measurements over at least 200 data points per sample. *t*-test was employed to assess statistical significance of the results. $P < 0.05$ was considered statistically significant.

2.1.4. Evaluation of bisphosphonate content

Bisphosphonate content was determined spectrophotometrically via complex formation with Fe(III) ions using a Varian Cary50Bio instrument (λ = 290 nm) [27].

2.1.5. Zeta potential

Zeta potential was measured using a Malvern Instruments Zetasizer Nano. 5 mg of powder sample was suspended in 50 mL of MilliQ water after sonication for 2 min. Spontaneous pH of all suspensions was about 8.5. Each analysis was performed in triplicate.

2.1.6. Hydrolysis

Hydrolysis tests were carried out on 100 mg of powders in 50 ml of 0.04 M HEPES solution at pH of 7.4, adjusted with NaOH. The solutions were stored at 60 °C for a period of time from 2 days up to 30 days.

2.1.7. Bisphosphonate release

Release tests were performed on disk-shaped samples (Ø = 6.0 mm). Each disk was prepared by pressing 60 mg of powder