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Synthesis and characterization of strontium-substituted hydroxyapatite nanoparticles for bone regeneration

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ABSTRACT

The production of stable suspensions of strontium-substituted hydroxyapatite (Sr-HA) nanopowders, as Sr ions vector for bone tissue regeneration, was carried out in the present work. Sr-HA nanopowders were synthesized via aqueous precipitation methods using Sr^{2+} amount from 0 to 100 mol% and were characterized by several complementary techniques such as solid-state Nuclear Magnetic Resonance spectroscopy, X-ray diffraction, Infrared spectroscopy, N_2 physisorption and Transmission Electron Microscopy.

The substitution of Ca^{2+} with Sr^{2+} in HA is always isomorphic with gradual evolution between the two limit compositions (containing 100% Ca and 100% Sr), this pointing out the homogeneity of the synthesized nanopowders and the complete solubility of strontium in HA lattice. Strontium addition is responsible for an increasing c/a ratio in the triclinic unit cell. A significant variation of the nanopowders shape and dimension is also observed, a preferential growth along the c-axis direction being evident at higher strontium loads. Modifications in the local chemical environment of phosphate and hydroxyl groups in the apatite lattice are also observed.

Stable suspensions were produced by dispersing the synthesized nanopowders in bovine serum albumin. Characterization by Dynamic Light Scattering and ζ-potential determination allowed to show that $Ca^{2+} \rightarrow Sr^{2+}$ substitution influences the hydrodynamic diameter, which is always twice the particles size determined by TEM, the nanoparticles being always negatively charged as a result from the albumin rearrangement upon the interaction with nanoparticles surface.

The biocompatibility of the suspensions was studied in terms of cell viability, apoptosis, proliferation and morphology, using osteosarcoma cell line SAOS-2. The data pointed out an increased cell proliferation for HA nanoparticles containing larger Sr^{2+} load, the cells morphology remaining essentially unaffected.

1. Introduction

Calcium phosphate ceramics, e.g. hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (HA) and tricalcium phosphate $Ca_3(PO_4)_2$ (TCP), are widely employed in the field of bone tissue engineering due to their controlled biodegradability and excellent biocompatibility [1–4]. Pure HA possesses a bi-pyramidal hexagonal crystal structure (space group $P6_3/m$, $a = b = 9.418$ Å, $c = 6.884$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$) where PO_4^{3-} tetrahedrons are regularly placed on two basal planes at 1/4 and 3/4 of the c-axis. Considering a unit cell, ten Ca^{2+} ions are located within two non-equivalent interstitial sites, four M(1) sites aligned to the c-axis at the cell edges and six M(2) sites forming two staggered equilateral triangles, placed above the phosphate basal plane. Within this triangular channel along the *c-axis* the two OH^- groups are placed [5].

The mineralized phase of bones, as well as enamel and dentin, is associated to nano-sized crystallites of calcium deficient HA [6], partially enriched by a large variety of substitutional ions. For example, one of the most common isomorphic ion replacement consists in the carbonatation of HA through the substitution of PO_4^{3-} or OH^- by CO_3^{2-} groups, up to ~7%wt [7,8]. In addition, biological apatites can also rearrange the presence of Mg^{2+}, Sr^{2+}, F^-, Cl^- or HPO_4^{2-} [9–12]

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within their crystal structure, influencing the biological behaviour of the material. In particular, strontium is strictly related to the bone response to osteoporosis, by promoting new tissue growth and decreasing its resorption [13].

Most studies point out that the incorporation of Sr in the bone occurs quite rapidly (within the first two weeks) [14]; X-ray diffraction analyses (XRD) show the distortions of the bone mineral crystals unit cell caused by Sr, confirming the incorporation of such alkaline ion into the crystalline lattice. Since then, after the development of the promising drug strontium ranelate (SR) (Protelos®, Servier), different types of studies were performed from mapping Sr in bones and teeth, to studying the incorporation of Sr into bone mineral (in particular in the crystal surface and lattice) and the decrease in calcium content, up to evaluate the effects of Sr in synthetic HA [15]. SR is currently used in the treatment of post-menopausal osteoporosis [16], although its prescription has been recently restricted to the acute medical cases because of cardiovascular side effects [17]. SR shows a dual beneficial action, enhancing the pre-osteoblastic cells differentiation and inhibiting the osteoclastic cells formation and functionality [18].

In a previous work [19], strontium has been shown to completely replace calcium ions in both non-equivalent interstitial sites. Due to the ionic radius difference ($Ca^{2+} = 0.100$ nm, $Sr^{2+} = 0.118$ nm), the presence of strontium results in a general perturbation of the lattice, increasing the cell parameters and modifying the mean size of the crystal domains [20]. In addition, previous in vitro and in vivo studies of the biological reactivity of Sr-substituted HA embedded in scaffolds like coatings on titanium components [21–23], gels [24], membranes [25] or tablets [26] in terms of cell vitality, proliferation and morphology have shown the osteogenic effect of Sr-HA. If these studies are relevant because they present potential effects that the incorporation of Sr could have on bone mineral, so far no studies were performed to develop and characterize stable suspension of strontium-substituted HA nanoparticles as a vector for Sr ions delivery for bone tissue engineering applications. For in vitro biocompatibility studies, a suitable agent must be selected to produce stable aqueous suspensions, avoiding nanoparticles flocculation [27,28] and growth by Ostwald ripening-like process. In addition, the dispersant should not have any effect on the test system, such as cell lines, microorganisms and animals. Bovine serum albumin (BSA) is a biological and mimic fluid and in a previous study was used as dispersant agent to obtain stable nanoparticles suspension [29], preventing toxic effects due to powder agglomeration. Therefore, in this work, BSA has been used for preparing nanoparticles suspension for biocompatibility evaluation.

The aim of the present work was to synthesize Sr-doped HA nanopowders and to investigate the effect of Sr presence within the apatite lattice by a detailed chemical, structural and morphological characterization of the powders. The synthesized Sr-doped HA nanopowders were then used to produce stable suspensions, analyse their efficiency on osteoblast viability and understand their suitability as nano-carriers for Sr delivery in the stimulation of bone tissue regeneration.

2. Materials and methods

2.1. HA nanopowders: synthesis and suspensions preparation

HA nanopowders containing various Sr^{2+} amount were synthesized by aqueous precipitation method, as reported in a previous work by Bigi et al. [19]. High-purity calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$, 99%w, Sigma Aldrich A.C.S. reagent), strontium nitrate anhydrous ($Sr(NO_3)_2$, 98%w, Alfa Aesar) and ammonium phosphate dibasic ($(NH_4)_2HPO_4$, >99.0%w, Fluka), were respectively used to prepare the Ca + Sr nitrate solutions (50 mL, 1.08 M overall) and the phosphate solutions (50 mL, 0.65 M), adjusted at pH 10 with NH_4OH (30%v). The synthesis was carried out in N_2 constant flow, to avoid as much as possible the carbonatation phenomena, adding drop-wise the phosphate solution to the nitrate solution at 90 °C under stirring. The solution was stirred at 90 °C in N_2 static atmosphere for 5 h; then, the white precipitate was centrifuged three times (10,000 rpm for 10 min), washed and finally dried at 80 °C overnight. The expected reaction is:

$$(10-x)Ca(NO_3)_2 \cdot 4H_2O + xSr(NO_3)_2 + 6(NH_4)_2HPO_4 + 8NH_4OH \rightarrow Ca_{10-x}Sr_x(PO_4)_6(OH)_2 + 20NH_4NO_3 + (46-4x)H_2O \quad (1)$$

Strontium content in the final HA powder was tailored changing the relative amount of $Ca(NO_3)_2{\cdot}4H_2O$ and $Sr(NO_3)_2$ in the nitrate solutions, to obtain a Sr/(Sr + Ca) molar ratio of 0, 5, 10, 25, 50, 75 and 100%; pure HA and Sr-doped HA nanopowders were labelled as Ca100 and SrX (X = molar amount of Sr), respectively.

To establish the best ratio between albumin and powder, different concentrations were tested. The best preparation was obtained by adding 5 mg of powder to 4 mL of 5% BSA (Sigma-Aldrich) aqueous solution. The suspension was sonicated for 1 h at 45 °C using LBS2 sonicator bath (FALC Instruments) with an operation frequency of 40 kHz and then diluted with Phosphate Buffer Solution (PBS). The obtained suspensions were labelled adding the suffix "S" to the powder label (e.g., SrXS).

2.2. Characterization techniques and procedures

2.2.1. Inductively coupled plasma optical emission spectrometry (ICP-OES)

The synthesized powders purity and composition were determined by ICP-OES (Spectro Ciros Vision CCD, 125–770 nm) using hydroxyapatite ultrapure standard (Reagent Grade, Sigma-Aldrich) and a 1000 ppm Sr standard (BHD SpectroSol). All samples and standards were dissolved in ultrapure nitric acid (70%v) and diluted in pure water from reverse osmosis (conductivity < 0.1 μS/cm), adding Cs (100 g/L) as ionization suppressor. The emission lines chosen for the analysis were 393.366 nm for Ca, 216.596 nm for Sr and 178.287 nm for P.

2.2.2. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were acquired in transmission mode using an Avatar Thermo FTIR spectrometer on KBr pellets in the range of 4000–400 cm^{-1} (resolution = 4 cm^{-1}, 64 scans). With the aim to investigate the interaction between nanopowders and bovine serum albumin (BSA), ATR-FTIR spectra were recorded on the suspensions previously lyophilized. Physical mixtures were also prepared merely blending 5 mg of each synthesized nanopowder with 200 mg of BSA to be compared with the data collected on the suspensions. ATR-FTIR spectra were recorded in the 4000–650 cm^{-1} range (resolution = 4 cm^{-1}, 256 scans) using a Nicolet FT-IR iS10 Spectrometer (Nicolet, Madison, WI, USA) equipped with a ZnSe plate ATR (Attenuated Total Reflectance) sampling accessory.

2.2.3. Nitrogen sorption

N_2 physisorption analyses were carried out on a Micromeritics ASAP 2010 analyser. Specific surface area (SSA) and volume pore distributions were calculated from N_2 adsorption/desorption isotherms applying BET equation and BJH model, respectively.

2.2.4. X-ray diffraction (XRD)

The mineralogical composition of the synthesized Sr-HA powders was analysed by XRD using a Rigaku DMAX III 4057A2 diffractometer, working at 40 kV and 30 mA (Cu K_α: 1.5418978 Å). All data were collected in the range $2\theta = 10°–60°$, with step size of 0.03° and dwell time of 10 s/step. Spectra were analysed by the Rietveld-method-based software MAUD (2.53 version), using PDF cards #09-0432 - Calcium-hydroxyapatite and #33-1348 - Strontium-hydroxyapatite as structural models.

2.2.5. Solid state nuclear magnetic resonance (NMR)

^{31}P and ^{1}H solid state NMR analysis were carried out with a Bruker 300WB instrument. Samples were packed in 4 mm ZrO_2 rotors, which