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Substitution of strontium and boron into hydroxyapatite crystals: Effect on physicochemical properties and biocompatibility with human Wharton-Jelly stem cells

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ABSTRACT

Hydroxyapatite (HA) enriched with strontium and boron ions was synthesized using two different methods: the precipitation method (Sr,B-HAw) and the dry method (Sr,B-HAd). Additionally, for the sake of comparison, the "pure" unsubstituted HA was prepared together with HAs substituted only with one type of a foreign ion. The obtained materials were subjected to physicochemical analysis with the use of various analytical methods, such as powder X-ray diffraction (PXRD), transmission electron microscopy (TEM), inductively coupled plasma optical emission spectroscopy (ICP-OES), Fourier transform infrared spectroscopy (FT-IR) and solid-state proton nuclear magnetic resonance (^{1}H ssNMR). All the obtained materials were also biologically tested for their potential cytotoxicity. The obtained materials (Sr,B-HAw and Sr,B-HAd) were homogeneous and respectively showed nano- and microcrystal apatitic structures. The simultaneous introduction of Sr^{2+} and BO_3^{3-} ions turned out to be more effective in respect of the dry method. Of importance, doped materials obtained using both synthesis routes have been demonstrated to be biocompatible, opening the way for medical applications.

1. Introduction

Calcium phosphates have been commonly used for many years as bone substitute materials for orthopaedics, dentistry or reconstructive surgery. This wide range of applications is possible thanks to the following qualities: capacity to form bonds with bone tissue, ability to form a scaffold for a newly formed bone, and facilitation of its growth [1,2].

The most commonly used calcium phosphate is hydroxyapatite (HA). Stoichiometric HA with $Ca_{10}(PO_4)_6(OH)_2$ formula shows a great similarity to biological apatite, which is the main inorganic component of mineralized tissues (i.e., bones, dentin and enamel).

Biomaterials engineering uses HA's susceptibility to partial ionic substitutions. According to the data provided by the literature, both Ca^{2+} cations and anions - orthophosphates (PO_4^{3-}) and hydroxyl (OH^-) - can be substituted [3,4]. Introduction of "foreign" ions to the HA structure may change its physicochemical and mechanical properties, as well as imparting additional biological properties, which is of extreme importance [3,5,6]. For instance, the introduction of copper or silver ions in place of calcium ions causes results in additional antibacterial activity with regard to apatite material [6,7]. On the other hand, the introduction of silicate ions has a favourable effect on the growth and development of bone tissue [8].

We aimed to obtain material enriched with strontium and boron ions.

Strontium has been clinically tested to have a favourable effect on bone tissue. In some countries, it is used in osteoporosis treatment and preventive care in women in the postmenopausal period [9,10]. Strontium ions at low concentrations are characterized with the bidirectional action: on the one hand, showing the capacity towards inhibiting the resorption process and, on the other hand, inducing bone formation by increasing osteoblast replication [11].

Boron is a microelement, which, at a molecular level, plays an important role in the bone metabolism process and is indispensable for proper regulation of body calcium balance, in turn preventing skeletal loss and protecting the organism from the development of osteoporosis [12,13].

There have been many articles dedicated to the synthesis and evaluation of HAs enriched with strontium ions [14–19]; however, there have only been a few concerning apatites substituted with boron ions [20–23]. In the available literature, we have been unable to find articles concerning HA simultaneously enriched with both these ions.

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Therefore, our main aim was to synthesize the material containing Sr^{2+} ions and BO_3^{3-} ions, then compare its properties with pure HA and HA containing strontium and boron solely.

For the physicochemical analysis, appropriate methods for precise descriptions of the powder materials were used, such as powder X-ray diffractometry (PXRD), Fourier transform infrared spectroscopy (FT-IR), solid-state proton nuclear magnetic resonance (ssNMR), and transmission electron microscopy (TEM). The elementary analysis was carried out with the inductively coupled plasma optical emission spectroscopy (ICP-OES) method. Additionally, the analysed materials were biologically tested with regard to cytotoxicity.

2. Materials and methods

2.1. Synthesis

Synthesis of the materials was facilitated using two distinctly different approaches: the standard precipitation method and the dry method. Each method (wet: w; dry: d) was used to obtain four different materials:

- Pure HA (HAw and HAd) with nominal composition $Ca_{10}(PO_4)_6(OH)_2$.
- HAs doped with strontium ions (Sr-HAw and Sr-HAd) with nominal composition $Ca_9Sr(PO_4)_6(OH)_2$.
- HAs doped with borate ions (B-HAw and B-HAd) with nominal composition $Ca_{10}(PO_4)_5(BO_3)(OH)_2$.
- HAs doped with both strontium and boron ions (Sr,B-HAw and Sr,B-HAd) with nominal composition $Ca_9Sr(PO_4)_5(BO_3)(OH)_2$.

a) Synthesis by the wet method

HAw and a series of substituted HA (Sr-HAw, B-HAw and Sr,B-HAw) were prepared by the standard wet method using $Ca(NO_3)_2 \cdot 4H_2O$, $(NH_4)_2HPO_4$, H_3BO_3 and $Sr(NO_3)_2$ as starting materials [24,25]. The amount of reagents was calculated on the assumption that strontium and borate ions would substitute calcium and phosphates, respectively.

Briefly, the solution of $(NH_4)_2HPO_4$ (or $(NH_4)_2HPO_4$ and H_3BO_3) was added dropwise to the solution of $Ca(NO_3)_2$ (or $Ca(NO_3)_2$ and $Sr(NO_3)_2$). The pH was adjusted to 9 with the addition of ammonium hydroxide solution. Upon complete addition of the reagents, the mixtures were stirred for 4 h at 60–70 °C, then aged at room temperature for 48 h. The resulting raw precipitates were filtered, washed several times with distilled water and then dried at 130 °C for 24 h.

b) Synthesis by the dry method

In the second method, apatitic samples (hydroxyapatite (HAd), Sr-substituted HA (Sr-HAd), B-substituted HA (B-HAd) and Sr,B-co-substituted HA (Sr,B-HAd) were synthesized using a solid state reaction method [20]. $CaCO_3$, $(NH_4)_2HPO_4$, H_3BO_3 and $SrCO_3$ were used as sources of calcium, phosphorus, boron and strontium, respectively. The starting materials were mixed carefully in an agate mortar with a defined molar proportion. The powders were then pressed into pellets under 5 T/cm^2, put into an alumina crucible and then heated in an electric furnace under air atmosphere as follows: at 450 °C for 12 h, 700 °C for 12 h and 1000 °C for 24 h. After calcination, the cooled pellets were pulverized.

2.2. Characterization

The phase composition of all the materials was determined by PXRD (Bruker D8 Discover diffractometer) using Cu Kα radiation ($\lambda = 1.54$ Å). The average crystallite sizes were calculated using the Scherrer formula. PXRD patterns were also used to determine the lattice constants by Rietveld refinement, which was processed using TOPAS software. The elemental strontium, boron, calcium and phosphorus contents were determined by ICP-OES (Perkin Elmer Optima 3100XL).

Apatitic crystals were observed using TEM (JEOL JEM 1400). For this purpose, a drop of a samples suspension in pure ethanol was placed on a Cu/formvar grid, dried and then measured under an accelerating voltage of 80 kV. In order to study the chemical structure of the obtained materials, middle infrared spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy in the solid state (ssNMR) were used. FTIR spectra were recorded in the 4000–400 cm^{-1} range using a PerkinElmer Spectrum 1000 spectrometer and pellets prepared by mixing and pressing together 2 mg of the sample and 200 mg of potassium bromide. All the infrared spectra were processed using the GRAMS/AI 8.0 software (Thermo Scientific).

The ^{1}H NMR experiments were performed on a Bruker WB 400 spectrometer, operated at 9.4 T, with Larmor frequencies of ^{1}H 400 MHz, respectively. Conventional one-pulse-acquire (Bloch -decay (BD)) spectra were measured with a 4 mm probe under magic angle spinning (MAS) at 7 kHz. The ^{1}H BD MAS NMR spectra were obtained with a $\pi/2$ ^{1}H pulse of 3.0 μs, 32 scans and a repetition time of 30 s.

2.3. Isolation and culture of Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs)

Human umbilical cord harvesting was approved ethically and methodologically by the local Research Institution and conducted among informed patients (written consent, non-opposition) in accordance with the usual ethical legal regulations (Article R 1243-57). All procedures were carried out in accordance with our authorization and registration number DC-2014-2262, which was allocated by the National *Cellule de Bioéthique*. Wharton's jelly-derived mesenchymal stem cells (WJMSCs) were enzymatically isolated from fresh human umbilical cords obtained after full-term births according to Mechiche Alami et al. [26]. WJMSCs were amplified at a density of 3×10^3 cell/cm^2 in an α-MEM culture medium (Lonza) supplemented with 10% decomplemented FBS (Dutscher), 1% penicillin/streptomycin/amphotericin B and 1% Glutamax® (*v*/v, Gibco), then maintained in a humidified atmosphere of 5% CO_2 at 37 °C with a medium change every two days. WJ-MSCs were seeded at 3000 cells/cm^2 in 24-well culture plates, which proliferated over three days. Powders were then added for three days at 1 mg per well after dry heat sterilization using a Poupinel furnace (Memert) at 250 °C for 2 h to get rid of any bacterial contaminant.

2.4. Cell viability

At the end of culture time, cell viability was assayed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) tests (CellTiter 96® AQueous One Solution Cell Proliferation Assay™, Promega). Cells were incubated with the reagent (100 μL in 500 μL of medium culture) for 1.5 h in a humidified atmosphere with 5% CO_2 at 37 °C. *medium* was collected and transferred to 96-well plates, after which the optical density was measured at 490 nm and corrected at 700 nm (Fluostar Omega, BMG Labtech).

2.5. Cell death

Cell death was determined by measuring lactate dehydrogenase (LDH) activity in cell culture supernatant at the end of culture time according to the manufacturer's protocol (Bio Vision Kit no. K311–400). The absorbance was read at 492 nm and corrected at 700 nm (Fluostar Omega, BMG Labtech).

2.6. Cell proliferation

Cell proliferation was assessed by DNA content assay. Total DNA was isolated from the cultures according to the protocol provided in the