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A biocompatible hybrid material with simultaneous calcium and strontium release capability for bone tissue repair

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ABSTRACT

The increasing interest in the effect of strontium in bone tissue repair has promoted the development of bioactive materials with strontium release capability. According to literature, hybrid materials based on the system PDMS–SiO_2 have been considered a plausible alternative as they present a mechanical behavior similar to the one of the human bone. The main purpose of this study was to obtain a biocompatible hybrid material with simultaneous calcium and strontium release capability. A hybrid material, in the system PDMS–SiO_2–CaO–SrO, was prepared with the incorporation of 0.05 mol of titanium per mol of SiO_2. Calcium and strontium were added using the respective acetates as sources, following a sol–gel technique previously developed by the present authors. The obtained samples were characterized by FT-IR, solid-state NMR, and SAXS, and surface roughness was analyzed by 3D optical profilometry. In vitro studies were performed by immersion of the samples in Kokubo's SBF for different periods of time, in order to determine the bioactive potential of these hybrids. Surfaces of the immersed samples were observed by SEM, EDS and PIXE, showing the formation of calcium phosphate precipitates. Supernatants were analyzed by ICP, revealing the capability of the material to simultaneously fix phosphorus ions and to release calcium and strontium, in a concentration range within the values reported as suitable for the induction of the bone tissue repair. The material demonstrated to be cytocompatible when tested with MG63 osteoblastic cells, exhibiting an inductive effect on cell proliferation and alkaline phosphatase activity.
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1. Introduction

Hybrid materials have the advantage of possessing properties that go beyond the simple sum of their organic and inorganic contributions [1]. A documented example is the combination of the mechanical properties of non-bioactive silicone (polydimethylsiloxane — PDMS), with the ability to bind to hard tissues of bioglasses [2,3], in an attempt to develop new materials to treat bone injuries/defects. The sol–gel processing at low temperatures of these hybrids is easy and has the capability to achieve monolithic parts. However it inhibits the use of precursors that require a firing/decomposition step to eliminate potentially toxic residues. During years this was a problem when dealing with the incorporation of calcium, an element of bioglass compositions associated with the biocompatibility. Calcium has been usually added to the sol–gel process using its nitrate form with a decomposition temperature higher than the degradation temperature of PDMS [4]. Since this problem was solved with the use of calcium acetate as a precursor [5], new possibilities opened.

With respect to the bone tissue repair, it is known that the bone formation/resorption equilibrium is affected by a variety of physical/chemical agents and processes such as inorganic ions, growth factors, hormones and stress actions [6].

The knowledge acquired regarding the role of ions, such as calcium, phosphorous, silicon, strontium, zinc, etc., in the stimulation of metabolic effects that occurs during tissue formation has shown the usefulness of these ions as therapeutic agents, after being incorporated in the composition of new biomaterials [4].

Strontium has demonstrated an in vitro stimulatory effect on osteoblasts together with an inhibitory effect on osteoclasts [7–9], and the ability to substitute calcium in the hydroxyapatite crystal lattice [8,10]. It has also been incorporated in the structure of new bioactive materials [11], and is used as a drug in the form of strontium ranelate to increase the densification of bone in osteoporotic patients [12]. Pharmacological studies about strontium ranelate reveal that it increases the alkaline phosphatase activity (a marker of osteoblast differentiation) in osteoblast precursor cells and in mature osteoblastic cells, and collagen

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synthesis (a marker of osteoblast function) [13]. In vivo studies of Sr-bioactive glasses [14] have shown that strontium has a dual function within bone remodeling. It is able to uncouple the process of bone resorption and bone formation by inhibiting osteoclasts and stimulating osteoblasts, respectively, leading to a gain in bone mass. Such findings heightened the need for the development of new bioactive materials with strontium release capability.

In the present work a hybrid material, in the system PDMS–SiO_2–TiO_2–CaO–SrO, was prepared. As far as it is known it is the first time that a sol–gel protocol is designed to add both calcium and strontium to the PDMS–SiO_2 hybrid system and its cytocompatibility is evaluated. Based on the authors' previous findings [15] the addition of titanium is justified by its capability to condition the sol–gel chemistry of the system PDMS–SiO_2, allowing the modulation of the final microstructure, besides the fact that titanium can also increase the bioactivity, due to surface Ti-OH groups [16]. To avoid the use of nitrates, calcium and strontium were added using the respective acetates as sources, according to a sol–gel technique [5].

2. Materials and methods

2.1. Sample preparation

Tetraethyl orthosilicate (TEOS), polydimethylsiloxane (PDMS) silanol terminated (550 g mol^{-1} average molecular weight), isopropanol (IPA), calcium acetate monohydrate ($Ca(CH_3CO_2)_2 \cdot H_2O$), strontium acetate ($Sr(CH_3CO_2)_2$), titanium isopropoxide (TiPr), all from Sigma-Aldrich, and ethyl acetoacetate (EtAcAc) from Merck, were used as raw materials for the preparation of the hybrids. Samples were prepared using a 1:0.05:0.05:0.18:0.05:5:0.22 TEOS/Ca-acetate/Sr-acetate/PDMS/TiPr/H_2O/HCl molar ratio. Separate aqueous solutions of $Ca(CH_3CO_2)_2 \cdot H_2O$ and $Sr(CH_3CO_2)_2$ (only the necessary amount of water was used to dissolve the acetates) were added to a mixture of TEOS, PDMS and the remaining H_2O [5]. Afterwards, isopropanol was added and the medium was acidified with HCl. The prepared solutions were then stirred for 2 h. After that TiPr, previously chelated with EtAcAc using a 1:2 propoxide/chelating agent molar ratio, was added. The final mixture was then stirred for another 3 h at room temperature and then poured into 15 mm diameter polyethylene cylindrical containers. The preparation was kept for a week at room temperature for gelation and then placed in an oven at 60 °C during another week for aging. After that the gels were dried at 150 °C for 24 h. Monolithic samples in the shape of cylinders were obtained without visible cracks. Cylinders were cut into discs with a diameter of ca. 15 mm and thickness of ca. 1.5 mm using a Struers Secotom-10 cutting machine. The preparation was named C5S5 regarding calcium and strontium Ca/TEOS and Sr/TEOS molar ratios × 100. For a better understanding of the influence of substituting calcium by strontium in the hybrid composition, the results obtained with an equivalent composition with a 0.10 Ca/TEOS molar ratio and no strontium, named here C10, were used for comparison.

2.2. Sample characterization

The sample structures were analyzed by FT-IR spectroscopy, ^1H MAS (magical angle spinning), ^{29}Si MAS and ^{29}Si–{^1H} CP-MAS (cross-polarization magical angle spinning) NMR, and SAXS, using the experimental conditions described in a previous work [15]. The surface topography of sample discs was evaluated using a 3D optical variation system IFM G4 (Alcona). Line and area measurements were done according to the recommendations described by the ISO standards 4287, 4288 and 25178. The following parameters were measured: arithmetic mean deviation of the surface (Ra), root-mean-square deviation of the surface (Rq), maximum height of summits (Rp), maximum depth of valleys (Rv), and total height of the surface (Rt). In addition, the fractal dimension (Df) and the developed area ratio (Sdr, ratio between the interfacial and the projected area) were also calculated.

The potential bioactivity of the materials was evaluated in vitro by immersion of the samples in Kokubos's [17] simulated body fluid (SBF) for 3, 7 and 14 days. The surfaces of the dried samples after soaking in SBF were observed by scanning transmission electron microscopy (STEM) (Hitachi SU-70) using an accelerating voltage of 25 kV, equipped with an electron dispersive spectroscopy (EDS) apparatus (Bruker QUANTAX 400). The same surfaces were also analyzed by particle induced X-ray emission (PIXE) using an Oxford Microbeams OM150 type scanning nuclear microprobe setup and a 2 MeV proton beam generated by a 2.5 MV Van de Graaff accelerator. The proton beam was focused down to $3 \times 4\ \mu m^2$ and the beam raster scanned over the sample surface covering an area up to $2640 \times 2640\ \mu m^2$. PIXE spectra were collected using a 70 mm^2 Si(Li) X-ray detector. The system allows efficient elemental distribution mapping (using the characteristic X-rays of elements heavier than Si) from which particular areas or points can be selected for obtaining spectra for representative quantitative analysis. Operation and basic data manipulation, including elemental distribution mapping, was achieved through the OMDAQ software code, and quantitative analysis with the DAN32 program [18]. Further system details can be found elsewhere [19]. The concentrations of Ca, P, Sr and Ti in the supernatant liquid were determined by inductively coupled plasma (ICP) (Jobin-Yvon JY70 Plus spectrometer).

Cut discs were sterilized using a total gamma irradiation dose of 25 kGy, the dose usually recommended [20,21] to achieve a sterility assurance level (SAL) of 10^{-6} (the probability of a micro-organism to survive after the process) when the natural contamination level and microorganism types cannot be calculated, in a procedure already described [22].

2.3. Osteoblastic cytocompatibility

Human osteoblastic-like MG63 cells (ATCC number CRL-1427™), of passage 25, were cultured in α-MEM, supplemented with 10% fetal bovine serum, 50 $\mu g \cdot mL^{-1}$ ascorbic acid, 50 $\mu g \cdot mL^{-1}$ gentamicin and 2.5 $\mu g \cdot mL^{-1}$ fungizone, at 37 °C, in a humidified atmosphere of 5% CO_2 in air. For sub-culturing, the cell layer (at around 70–80% confluence) was detached with trypsin–EDTA solution (0.05% trypsin, 0.25% EDTA; 5 min, 37 °C). The cell suspension was used in the experiments.

Cells were seeded over the materials' samples, at a density of 2×10^4 $cells \cdot cm^{-2}$, and were cultured for 10 days. Cell behavior was characterized throughout the culture time for cell proliferation, alkaline phosphatase activity and observation by SEM.

2.3.1. DNA content

Cell proliferation was evaluated by analyzing the DNA content, using the PicoGreen DNA quantification assay (Quant-iT™ PicoGreenR dsDNA Assay Kit, Molecular Probes Inc., Eugene. At each time-point, culture medium was removed and the cultures were treated with Triton X-100 (Sigma, 0.1%, 5 min) to lyse the cell layer. DNA was assessed in the cellular lysates, according to the manufacturer's instructions. Fluorescence was measured on an ELISA reader (Synergy HT, Biotek) at wavelengths of 480 and 520 nm, excitation and emission respectively, and corrected for fluorescence of reagent blanks. The amount of DNA was calculated by extrapolating a standard curve obtained by running the assay with the given DNA standard.

2.3.2. Alkaline phosphatase (ALP) activity

ALP activity was evaluated in cell lysates (0.1% Triton X-100, 5 min) by the hydrolysis of *p*-nitrophenyl phosphate in alkaline buffer solution (pH ~ 10.3; 30 min, 37 °C) and colorimetric determination of the product (*p*-nitrophenol) at 400 nm in an ELISA plate reader (Synergy HT, Biotek). ALP activity was normalized to total protein content (quantified by Bradford's method) and was expressed as nmol/min·μg $protein^{-1}$.

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