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Camila B Tovani, Tamires M Oliveira, Mariana P R Soares, Nadine Nassif, Sandra y Fukada, et al.. Strontium Calcium Phosphate Nanotubes as Bioinspired Building Blocks for Bone Regeneration. ACS Applied Materials & Interfaces, 2020, 12 (39), pp.43422 - 43434. 10.1021/acsami.0c12434 . hal-03004581

HAL Id: hal-03004581

<https://hal.science/hal-03004581>

Submitted on 13 Nov 2020

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ACS Appl. Mater. Interfaces, **Just Accepted Manuscript** • DOI: 10.1021/acsami.0c12434 • Publication Date (Web): 02 Sep 2020

Downloaded from pubs.acs.org on September 3, 2020

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Strontium-calcium phosphate nanotubes as bioinspired building blocks for bone regeneration

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Keywords: biomineralization, calcium phosphate, strontium, collagen, confinement, nanotubes

Abstract

Calcium phosphates (CaPs) based ceramics are the most investigated materials for bone repairing and regeneration. However, the clinical performance of commercial ceramics is still far from that of the native tissue which remains as the gold standard. Thus, reproducing the structural architecture and composition of bone matrix should trigger biomimetic response in synthetic materials. Here, we propose an innovative strategy based on the use of track-etched membranes as physical confinement to produce collagen-free strontium substituted-CaPs nanotubes that tend to mimic the building block of bone, *i.e.* the mineralized collagen fibrils. A combination of high-resolution microscopic and spectroscopic techniques revealed the underlying mechanisms driving the nanotubes formation. Under confinement poorly crystalline apatite platelets assembled into tubes which resembled the mineralized collagen fibrils in terms of diameter and structure of bioapatite. Furthermore, the synergetic effect of Sr^{2+} and confinement gave rise to the stabilization of amorphous strontium-CaPs nanotubes. The nanotubes were tested in long-term culture of osteoblasts supporting their maturation and mineralization without eliciting any cytotoxicity. The Sr^{2+} released from the particles reduced the differentiation and activity of osteoclasts in a Sr^{2+} -concentration dependent manner. Their bioactivity was evaluated in a serum-like solution showing that the particles guided spatially the biomimetic remineralization. Further, these effects were achieved at strikingly low concentrations of Sr^{2+} that is crucial to avoid side effects. Overall, these results open simple and promising pathways to develop a new generation of CaPs multifunctional ceramics active in tissue regeneration and able to simultaneously induce biomimetic remineralization and control the imbalanced osteoclast activity responsible for bone-density loss.

1. INTRODUCTION

Producing materials able to conduct the cascade of events involved in tissue regeneration is an ongoing goal in biomaterials science. This requires reproducing the chemistry and structure of the native tissue.¹ In bone, the key mechanical and biological features arise mainly from the fibrillar pattern of the extracellular matrix.² Collagen molecules self-assemble into fibrils of 100-200 nm that are mineralized by apatite platelets providing a typical anisotropic environment that directs cell proliferation and differentiation.³ Bone mineral contributes not only for the mechanical properties of the tissue but also supports its remodeling process and biochemical function of ionic reservoir.⁴ This is ascribed to the low degree of crystallinity and the presence of amorphous domains that provides adequate resorption rate. Calcium phosphates (CaPs), are among the most extensively materials used to repair hard tissues injuries thanks to their chemical resemblance to the mineral phase of bone and teeth, suitable biocompatibility and low cost.⁵ However, producing biomimetic CaPs particles remains challenging. In contrast to bone mineral, CaPs based commercial ceramics are usually stoichiometric and highly crystalline rendering poor osteogenic functions as compared to biological graft.⁶ Consequently, rather than regenerative materials, the applications of CaPs are reduced to biologically passive replacers or fillers. Tuning the physicochemical properties is therefore crucial to trigger biomimetic response in synthetic materials.

To date, several strategies have been proposed to produce bioactive ceramics dedicated to bone regeneration, being the combination of CaPs with collagen regarded as one of the most effective approach to mimic the fibrillar nature of the tissue.^{7,8} However, working with collagen is hard to handle and expensive. Additionally, (i)

denaturation of collagen molecule is easily induced *in vitro* compromising its ability to self-assemble into fibrils and consequently the biocompatibility of the materials and (ii) reaching fibrils monodisperse in size is not straightforward and requires high concentrated collagen solutions which stabilization is challenging. One dimensional particles with tunable diameter offer an alternative pathway to mimic the mineralized collagen fibrils of bone. Apatite nano/micro tubes have been synthesized through several conditions including hydrothermal routes, high temperatures and addition of stabilizing agents.⁹ Considerable progress has been made, however simultaneously reproducing the structural features of bone mineral and the morphology of collagen fibrils is still challenging and no CaPs particles which resemble the environment of bone extracellular matrix have been achieved without the need of an organic scaffold.

Although biomimetic CaPs can repair damages, induce remineralization and osteogenesis, they are not able to regulate the increased osteoclastic activity responsible for osteoporosis.^{10,11} In this sense, in addition to tune the structure and morphology, the incorporation of Sr^{2+} in bioceramics represents a leap forward in the field of bone regeneration.¹² Unlike the traditional osteoporosis therapies, Sr^{2+} exerts a twofold effect on bone cells stimulating osteoblasts activity and decreasing osteoclasts metabolism simultaneously, as desirable for treating and preventing osteoporosis.¹³ Among the materials investigated to locally deliver Sr^{2+} , amorphous CaPs stand out. It is readily degraded by osteoclasts in the acidic environment during bone remodeling yielding both ionic precursors to remineralization and Sr^{2+} to regulate the imbalanced cellular activity.^{14,15} The use of amorphous CaPs for bone repairing is especially advantageous since they easily induce biomimetic remineralization.¹⁰ Besides the encouraging clinical results, one should emphasize that the side effects of Sr^{2+} cannot be neglected; its accumulation in the tissue leads to pathological mineralization such as rickets and

osteomalacia.^{16–18} Therefore, developing biomaterials able to reduce osteoclasts activity at low doses of Sr^{2+} is of paramount significance since elderly people who may present decreased renal function are the main target of osteoporosis treatments.

Here, we propose an innovative strategy based on the use of physical confinement to produce collagen-free CaP nanotubes which tend to mimic the building block of bone, the mineralized collagen fibrils. Under confinement, poorly crystalline apatite platelets assembled into tubes, which simultaneously resemble both the fibrils present in the extracellular matrix of bone in terms of diameter range and the structure of bioapatite. Although collagen is described to define the structural features of bone mineral, biomimetic apatite was formed here only by using physical confinement, discarding the use of fibrillar polymers. The incorporation of Sr^{2+} changed the mineral phase from apatite to amorphous CaP and strontium apatite. The Sr^{2+} released from the particles in the cell culture medium increased alkaline phosphatase activity of osteoblasts and decreased osteoclasts differentiation and resorption in a Sr^{2+} concentration dependent manner. Notably, these effects were reached at extremely low amounts of Sr^{2+} as compared to typical therapies based on strontium ranelate and currently reported biomaterials.^{19–21} The ability of the particles to promote biomimetic remineralization was demonstrated by the deposition of bone-like apatite on their surface after immersion in a simulated body fluid (SBF). These results evidence that the particles synthesized herein open perspectives for new ceramics towards bone regeneration.

2. EXPERIMENTAL PROCEDURE

2.1 Formation of strontium calcium phosphate Sr(CaP) nanotubes in physical confinement

Bioinspired precipitation of CaPs containing different amounts of Sr^{2+} (Sr(CaP)) was carried out in confined environment provided by the 200 nm cylindrical pores of commercial hydrophilic polycarbonate track-etched membranes (Millipore®) of 20 μm thickness, by modifying an approach described elsewhere.²² The size of pores was chosen based on the diameter of collagen fibrils in bone. Briefly, (0.1 wt.% poly(acrylic acid) (PAA, Sigma MW1800 g mol^{-1}) was dissolved in aqueous solutions containing 0.006 mol L^{-1} H_3PO_4 (Sigma), CaCl_2 (Merck P.A.), SrCl_2 (Synth P.A.), or a mixture of these salts at different Sr^{2+} molar percentages (0, 10, 50, and 100%) in relation to the total number of mols of divalent cations ($\text{Ca}^{2+} + \text{Sr}^{2+}$). The total $[\text{Ca}^{2+}] + [\text{Sr}^{2+}]$ concentration was 0.01 mol L^{-1} . The (Ca + Sr)/P molar ratio was kept constant and equal to 1.67 and initial pH = 3.5. The membranes were initially plasma cleaned (Harrick-plasma chamber) using $\text{N}_2(\text{g})$ for 2 min. In the sequence, they were immersed into the PAA/salt solution for 12 h allowing the penetration of the solution within the pores of the membrane. PAA is described to increase the synthesis yield by promoting the formation of the so-called mineral precursor phase PILP (polymer induced liquid precursor phase) which displays the liquid ability to infiltrate within restricted volumes by capillary action enhancing significantly the intra-membrane *versus* bulk precipitation.²³ The membranes were subsequently placed between two glass slides and put into a desiccator containing $(\text{NH}_4)_2\text{CO}_3$ at room temperature for 12h, as schematically represented in Figure 1. The $(\text{NH}_4)_2\text{CO}_3$ decomposition generates $\text{CO}_2(\text{g})$ and $\text{NH}_3(\text{g})$ that diffuse into the aqueous solutions increasing the pH which triggers the

1 mineral precipitation within the pores of the membrane. After precipitation, the surface
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1 mineral precipitation within the pores of the membrane. After precipitation, the surface
2 of the membranes was scraped using filter paper to remove particles that were not
3 formed within the pores. To recover the nanotubes, the membranes were dissolved with
4 chloroform followed by centrifugation (11000 rpm) three times. The particles were then
5 rinsed with ethanol and characterized.

6 7 **2.2 Precipitation from the bulk**

8 Precipitation from bulk solution was carried out as control experiments. To this
9 end, 50-mL flasks containing 20 mL of the PAA/salt starting solutions were placed in a
10 closed desiccator under identical reaction conditions used for the intramembrane
11 precipitation, *i.e.* precursors concentration, temperature, dissector volume and reaction
12 time. The precipitates were centrifugated, rinsed with ethanol and dried at room
13 temperature.

Figure 1. (a) Schematic representation of a track-etched membrane used as physical confinement to tune the morphology and size of the nanotubes. The side and top view of the membrane show the cylindrical pores where the precipitation takes place. A SEM image of the surface of a membrane shows the pores. (b) Schematic representation of the precipitation of Sr(CaP) under either physical confinement (i, ii, iv) or in bulk solution (iii and v). The membranes were immersed into the PAA/salt solution for 12 h (i) and then placed in a desiccator containing $(\text{NH}_4)_2\text{CO}_3$ (ii) leading to the formation of nanotubes inside the cylindrical pores (iv). The particles (reddish SEM image) were isolated by dissolving the membranes with chloroform followed by centrifugation. A beaker containing the PAA/salt solutions was placed in the desiccator (iii) to conduct the precipitation from bulk solution (v) leading to agglomerated spherical particles (greenish SEM image).

2.3 Ability of the particles to induce biomimetic mineralization *in vitro*

The particles were dispersed in simulated body fluid (SBF) at 37°C for 5 days to evaluate their ability to induce biomimetic precipitation of apatite in physiological environment. The procedure used to prepare SBF followed the International Standard Organization (ISO 23317) and is described elsewhere.²⁴ Briefly, 2.5 mmol L⁻¹ Ca²⁺, 142 mmol L⁻¹ Na⁺, 4.2 mmol L⁻¹ HCO₃⁻, 5 mmol L⁻¹ K⁺, 1.5 mmol L⁻¹ Mg²⁺, 147.8 mmol L⁻¹ Cl⁻, 1 mmol L⁻¹ HPO₄²⁻ and 0.5 mmol L⁻¹ SO₄²⁻ were dissolved in ultrapure water. The pH was adjusted to 7.4 with 11 mol L⁻¹ tris-hydroxymethyl amine methane (Tris) and 1 mol L⁻¹ HCl aqueous solution.²⁴

2.4 Characterization of the samples

The morphology of the gold-coated particles was investigated by scanning electron microscopy (SEM) using a Zeiss-EVO 50 microscope under 20 kV accelerating voltage. For transmission electron microscopy (TEM) analysis, the samples were dispersed in ethanol and some drops were deposited on a lacey carbon film on copper grid. Energy dispersive spectroscopy (EDS) mapping of Ca, Sr and P was obtained on a single particle using a HRTEM microscope FEI TECNAI G2 F20 operating at 200 kV. TEM images and single particle selected area electron diffraction (SAED) patterns were acquired using a TEM microscope JEOL 2011 operating at 100 kV. Energy electron loss spectra (EELS) and scanning transmission electron microscope images (STEM) were acquired in a Nion STEM microscope at 200 keV. The chemical groups were identified by Fourier-transform infrared spectroscopy (FTIR) coupled with an attenuated total reflectance (ATR) accessory (Shimadzu-IRPrestige-21), with a resolution of 2 cm⁻¹. The X-ray diffraction patterns were acquired with a Bruker-AXS

D5005 diffractometer using Cu-K α radiation at 40 kV and 30 mA. The diffraction peaks were indexed based on the databank of the Joint Committee on Powder Diffraction. The electrophoretic mobility measurements for the determination of zeta-potential (ζ) were carried out in a Zetasizer Nano ZS (Malvern Instruments). For evaluation of ζ as a function of the pH, 1 mg mL $^{-1}$ of particles was dispersed in 0.001 mol L $^{-1}$ NaCl aqueous solution to keep constant the ionic strength. The pH was adjusted using 1 mol L $^{-1}$ HCl and 1 mol L $^{-1}$ NaOH. ζ *versus* pH curves were also obtained to investigate the behavior of the particles in the cell culture medium. To this end, the particles were incubated with minimum essential medium (α -MEM, Gibco) supplemented with 10 wt.% fetal bovine serum for 30 min. In the sequence the particles were centrifugated and gently washed with deionized water followed by redispersion in 0.001 mol L $^{-1}$ NaCl aqueous solution.

2.5 Release of Sr $^{2+}$

The Sr $^{2+}$ released from the particles after immersion in cell culture medium for 1, 3, 7, 14 and 21 days was quantified by atomic absorption spectrometry with atomization by acetylene-air flame in the equipment ContrAA 700 (Analytik Jena AG, Jena, Germany). The samples were supplemented with La $^{3+}$ (10 g L $^{-1}$) and K $^{+}$ (1 g L $^{-1}$) and the absorbance read at 460 and 733 nm.

2.6 Cellular response *in vitro*

2.6.1 MC3T3-E1 viability and mineralization ability

Osteoblastic lineage cells MC3T3-E1 (American Type Culture Collection-ATCC) were cultured in α -MEM (Gibco) supplemented with 10 wt.% fetal bovine serum and 1 vol.% penicillin/streptomycin. This is a lineage with an osteoblastic

1 phenotype that undergoes a proliferation–differentiation sequence, leading to the
2 mineralization of bone-like extracellular matrix. The osteogenic medium was achieved
3 by the addition of ascorbic acid and β -glycerophosphate. Briefly, the cells were seeded
4 on 24-well plates at the density of $2 \cdot 10^4$ cells per well and incubated in air at 37 °C and
5 5% CO₂. The plated cells were then allowed to attach to the polystyrene well bottoms
6 for 24h, followed by the replacement of the medium for a
7 $1 \mu\text{g mL}^{-1}$ suspension of nanotubes (0% Sr²⁺, 10% Sr²⁺, 50% Sr²⁺ and 100% Sr²⁺) in the
8 culture medium. The medium was changed every two days keeping constant the
9 concentration of the particles. Cell viability was determined by MTT (3(4,5-dimethyl-
10 thiazole-2-yl)2,5-diphenyl tetrazolium bromide, Sigma-Aldrich) assay after 7, 14 and 21
11 days of culture using the protocol described by Mosmann.²⁵ Cell viability was expressed
12 as the percentage of the average of 3 experiments as compared to the control (Ct)
13 without treatment, for each day of culture (100%). ALP activity was determined by
14 quantifying the hydrolysis of the substrate p-nitrophenyl phosphate (PNPP, Sigma-
15 Aldrich) in the plasma membrane fraction extracted from cells after 7 and 14 days of
16 culture, according to the protocol described elsewhere.²⁶ The enzymatic activity was
17 normalized by the total protein content which was estimated by the Hartree
18 methodology, in the presence of 2 wt.% sodium dodecyl sulfate and using bovine serum
19 albumin as standard.²⁷ To this end, the absorbance of the samples was read at 650 nm
20 with a spectrometer. After culturing for 21 days, the formation of the mineralized
21 extracellular matrix was investigated by Alizarin Red S (Sigma-Aldrich) staining
22 according to the methodology described elsewhere.²⁸ To avoid the interference of
23 Sr(CaP) particles, the wells were first gently washed with phosphate saline buffer (three
24 times) removing possible loosely-bounded precipitates from the culture medium. In the
25 sequence, the samples were fixed in 1.5 vol.% glutaraldehyde at 4 °C for 12 h and

dehydrated through a series of ethanol concentrations (30, 50, 70, and 100 vol.%). The content of the wells was solubilized in acetic acid and neutralized with ammonium hydroxide. The formation of mineral nodules was then followed by reading the absorbance of the samples at 405 nm.

2.6.2 Cell morphology

SEM was applied to investigate morphology of cells. The cells cultured on the polystyrene discs were fixed in 1.5 vol% glutaraldehyde at 4 °C for 12 h, and dehydrated through a series of ethanol concentrations (20, 50, 70, 80, 90 and 100 vol.%), dried using supercritical CO₂ conditions and contrasted with OsO₄. After coated with gold, the samples were observed on a Zeiss-EVO 50 SEM microscope under 20 kV accelerating voltage. The morphology of attached cells was also investigated by confocal microscopy. For this, after the fixation step, the samples were stained with 5 µg mL⁻¹ acridine orange (Sigma-Aldrich) and then visualized using a confocal laser scanning microscope (Leica TCS SP5). This fluorophore binds to the osteoblast nucleic acids providing a contrasted image. To visualize DNA, the sample was excited with the 488 nm line of an argon laser, and emission was collected between 499 and 541 nm (green). RNA was visualized by exciting the sample with the 458 nm line of an argon laser, and emission was collected between 642 and 682 nm (red).

2.6.3 Osteoclasts differentiation and resorption activity

Bone marrow macrophages (BMMs) were isolated from long bone of 6- to 8-wk-old C57BL/6 mice and were cultured in α-MEM (GIBCO, Invitrogen) supplemented with 10 wt.% fetal bovine serum (FBS; Sigma-Aldrich), 1 vol.% penicillin-streptomycin, and 30 ng mL⁻¹ M-CSF (R&D Systems). After 3 days, adherents BMMs were collected, seeded at a density of 2.10⁴ cells/well in 96-well plates or

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1 hydroxyapatite-coated plates. Cells were cultured for 2 days in osteoclastogenic
2 medium condition: M-CSF (30 ng mL⁻¹) and RANKL (10 ng mL⁻¹). Then, the
3 osteoclastogenic medium was replaced by 1 µg mL⁻¹ dispersion of the of Sr(CaP)
4 nanotubes containing 0, 10, 50, 100 mol% Sr²⁺ in the culture medium. Osteoclast-
5 differentiated cells on day 4 were confirmed by tartrate-resistant acid phosphatase
6 (TRAP) staining (Sigma-Aldrich 387A kit). The osteoclast number (TRAP-positive
7 cells containing 3 or more nuclei) was analyzed in 5 wells per group using ImageJ
8 software (National Institutes of Health). Resorption area was measured in
9 hydroxyapatite-coated 96 well plate (OsteoAssay-Corning) on day 5 of culture. The
10 results were representative of two independent experiments.

3. RESULTS AND DISCUSSION

3.1 Synthesis of Sr(CaP) nanotubes: effect of physical confinement and Sr^{2+} concentration

The effects of the physical confinement in the precipitation of Sr(CaP) particles were distinguished by comparison with control experiments in which the precipitation was carried out in bulk solution. SEM images of the polycarbonate track-etched membranes used as physical confinement are shown in Figure S1. The effect of Sr^{2+} concentration was also investigated. Polydisperse and agglomerated spherical particles formed in the control experiments (Figure 2a). The spherical morphology is typical of apatite grown in the presence of high concentration of CO_3^{2-} .²⁹ Well defined tubes formed within the pores of polycarbonate membranes regardless the amount of Sr^{2+} , confirming the role of physical confinement in defining the particles diameter and morphology (Figure 2b). Notably, these particles resemble the collagen fibrils found in the extracellular matrix of bone in terms of diameter range and mineral interface.³⁰ To demonstrate the versatility of this approach to control the morphology and size of the particles, Sr(CaP) tubes with 400 nm diameter were synthesized using polycarbonate membranes with 400 nm pores (Figure S2). The Sr^{2+} incorporation in the solids was proportional to its concentration in the starting solutions regardless the use of confinement, as determined by TEM-EDS (Table S1).

The mineral composition of the spherical particles precipitated in bulk solution and tubes precipitated under confinement was investigated by XRD (Figures 2c and 2d, respectively). In the absence of Sr^{2+} (0% Sr^{2+}) apatite was formed in the control together with other CaPs (indicated by * in the diffractogram). Although some peaks could be identified as octacalcium phosphate (OCP) the low amount of material precluded the

complete identification of these additional phases by XRD. Poorly crystalline apatite resembling bone mineral was selectively formed within the membrane pores at 0% Sr^{2+} .³¹ The addition of 10% Sr^{2+} in the control lead to strain in the crystalline structure of apatite as suggested by the broader diffraction peaks (Figure 2c). Moreover, the shift of the 002 peak towards lower values of 2θ supports the Ca^{2+} replacement by Sr^{2+} in the apatite lattice. At the same 10% Sr^{2+} , amorphous strontium calcium phosphate Sr(ACP) was formed under confinement (Figure 2d). At 50% Sr^{2+} , Sr(ACP) was precipitated in the control as indicated by the characteristic humps at 2θ close to 30° (Figure 2c), in agreement with previous investigations.³² Additionally, some peaks assigned to strontianite (SrCO_3 , indicated by S in the diffractogram) were also detected.³³ This was expected as the critical pH for the mineral precipitation was triggered by the decomposition of $(\text{NH}_4)_2\text{CO}_3$. In contrast, diffraction peaks were not observed for the samples containing 50% Sr^{2+} precipitated within the membrane's pores, showing that confinement promotes the amorphization of both phosphate and carbonate. This indicates therefore that this sample is formed by a mixture of Sr(ACP) and amorphous strontium calcium carbonate Sr(ACC). Increased amount of Sr^{2+} up to 100% resulted in the precipitation of strontium hydrogen phosphate for the control, while strontium apatite was formed under confinement. Table S2 summarizes the products obtained in bulk solution and in confinement. ζ results evidenced the structural changes described by XRD due to the incorporation of Sr^{2+} in the particles (Figure S3). ATR-FTIR spectra confirmed that, likewise the mineral found in the bone tissue and tooth enamel, the nanotubes are made of carbonated apatite, thus strengthening their biomimetic nature (Figure S4).³⁴ This is an important feature considering the application of these particles as building blocks for bone regeneration.

Figure 2. SEM images of the phosphate particles formed in bulk solution (a) and in confinement (b). XRD pattern of the particles synthesized in bulk solution (c) and in confinement (d). The products formed in bulk solution were indexed as follows: hydroxyapatite for 0% Sr²⁺ and 10% Sr²⁺ (JCPDS 9-432). The additional peaks (*) observed in the 0% Sr²⁺ sample may be due to OCP (JPDCS 26-1056). Strontianite (S) (JPDCS 01-071-4899) was found in the 50% Sr²⁺ sample in addition to an amorphous phase Sr(ACP). Strontium hydrogen phosphate was identified in the 100% Sr²⁺ sample (JCPDS 23-105026). The samples formed in confinement were identified as hydroxyapatite (0% Sr²⁺), Sr(ACP) (10% Sr²⁺ and 50% Sr²⁺) and strontium hydroxyapatite (100% Sr²⁺) (JPDCS 33-1348).

The tubes formed under physical confinement were further characterized by TEM-BF (bright field), STEM-HAADF (high angle annular dark field) microscopy and SAED (Figure 3). As expected, the tubes display a diameter of 200 nm. The samples containing 0, 10 and 50% Sr^{2+} displayed a hollow structure which characterized them as tubes. This was unambiguous from the STEM-HAADF images that were sensitive to the amount of projected materials with higher thickness appearing brighter. Furthermore, vase-like structures were observed suggesting that the precipitation started at the bottom of the membrane and progressed along the pore leading to narrowing tube walls (Figure S5). In the absence of Sr^{2+} , the tubes were polycrystalline composed of nanometric platelets resembling the morphology of apatite in bone. Their apatite structure was confirmed by the typical SAED patterns.

The absence of crystalline reflections in the SAED patterns of the 10% Sr^{2+} and 50% Sr^{2+} tubes confirms their amorphous character depicted by XRD. The 100% Sr^{2+} rods were polycrystalline, with grain sizes of several tens of nm and unlike the Ca^{2+} -bearing particles they were not hollow. Elementary EDS-TEM mapping results show that the elements Ca, Sr, P, and O were homogeneously distributed within the Sr(ACP) particles, at least with the resolution of the EDS system (Figure S6).

Figure 3. TEM-BF, STEM-HAADF images and SAED patterns of the 0, 10, 50, and 100% Sr²⁺ nanotubes. STEM-HAADF images show that the 0% Sr²⁺, 10% Sr²⁺ and 50% Sr²⁺ nanotubes are hollow. The absence of crystalline reflections in the SAED patterns of the 10% Sr²⁺ and 50% Sr²⁺ nanotubes confirms their amorphous character depicted by XRD.

3.2 Nanoscale Analysis by Electron Energy Loss Spectromicroscopy (EELS)

The chemical composition of the nanotubes was investigated with nanometric resolution by EELS. STEM-EELS offers the advantage of spatial resolution and has been used as powerful technique in biomineralization and biomaterials investigations.

The spectra in Figure 4a exhibit the Sr-L (ca. 1950 eV) and P-K (ca. 2150 eV) edges averaged over typically 100 nm x 100 nm surface area. The Sr/P intensities drastically change as Ca^{2+} is substituted by Sr^{2+} in the samples. Quantifications can be done and typical Sr/P ratios of 0, 0.16, 0.8 and 1.7 are obtained in agreement with the expected composition within the 10% EELS accuracy. The EELS edges at lower energy, also obtained with ca. 100 nm of spatial resolution, are displayed in Figure 4b. A clear change of Ca L (ca. 350 eV) intensity is observed as the amount of Sr^{2+} increases in the samples. On the other hand, the P-L and Sr-M edges are overlapping giving a cumbersome situation where no reliable quantification can be done. STEM-EELS were also done with nanometer scale resolution by acquiring spectromicroscopic images or lines across the apparent walls of the hollow structures (Figure 4c). To achieve such spatial resolution, electron doses have to be increased and the overall SNR ratios of the EELS signal get lower. With such conditions, no quantifications have been done, even for the non-overlapping edges. We focus on the 50% Sr^{2+} amorphous nanotubes where heterogeneities have been robustly measured, notably on the sidewall of the tube. The analysis of Sr-L and P-K edges indicated that the outer surface of the tube (the surface that grows at the interface with the pore) is phosphorus enriched (Figure 4d). With the lower energy edges (figure 4e), it is not possible to differentiate between P and Sr, but comparing with the Ca-L distribution, heterogeneities are present throughout the tube wall. The Sr and Ca distribution do not have maximum intensity at

the same position and, in particular a clearer Ca enriched layer at the inner surface of the tube is observed. Furthermore, both surfaces show the presence of additional C-layers. Such carbon layers are often present in STEM-EELS experiments and might be due to contamination during the STEM experiments or the sample preparation. Additional EELS experiments have been done keeping the sample near LN₂ temperature, that is known to strongly reduce contamination and degradation issues, and similar carbon rich layers of several nm have been detected in the outer surface (Figure S7). The presence of carbonate groups can be observed by EELS since its spectroscopic C-K fine structure is different from the one of amorphous carbon (or contamination carbon or heavily damage organic carbon). As shown in Figure S7d, carbonate ions are present in the Sr(ACP), and at the Ca enriched layer at the inner surface. On the other hand, the carbon layer on the outer surface has a broader spectroscopic structure and might be the beam damaged PAA. These STEM-EELS results clearly evidence heterogeneities from the outer to the inner part of the hollow tubes whose might origin from the precipitation into confined mesoporous cavity.

Figure 4. (a,b) EELS spectra of the tubes containing 0%Sr²⁺, 10%Sr²⁺, 50%Sr²⁺ and 100%Sr²⁺. (c,d,e) EELS spectromicroscopy performed on the 50%Sr²⁺ nanotube showing the presence of several compositional heterogeneities.

3.3 Formation mechanism of Sr(CaP) nanotubes

From these results it can be clearly seen that Sr^{2+} and physical confinement have a marked effect over the formation CaP particles in terms of size, morphology, and selection of mineral phase. For instance, while the most reported morphology of ACP is spherical, here we were able to tailor the shape of Sr(ACP) into tubes thanks to the boundary offered by the membrane pores which in turn direct the mineral growth.³² Likewise, the nanometric apatite crystals precipitated in absence of Sr^{2+} assembled into tubes, rather than the usually spherulitic morphology.³¹ Interestingly, while collagen is reported to define the structural features of bone mineral, biomimetic apatite was formed here only by using physical confinement.

Recently, we have shown that the biomimetic precipitation of Sr(ACP) in homogeneous media starts when the degree of Ca^{2+} substitution by Sr^{2+} reaches 25% in the crystalline lattice of apatite.³² However, this amorphous phase was stabilized in the reaction medium only at higher degrees of Sr^{2+} substitution, namely 50 and 75%. Here, by controlling the physical environment where the precipitation takes place, Sr(ACP) was formed at lower amounts of Sr^{2+} , *i.e.* 10% Sr^{2+} , that is the maximum percentage of this ion found in the bone tissue.³⁵ EDX analysis confirmed that this effect was provided by the physical confinement since the amount of Sr^{2+} incorporated into the products formed in bulk and within the pores of the membranes was similar. Relying on the pathway of apatite formation *in vitro* and previous findings, we hypothesize that Sr^{2+} acts as kinetic stabilizing agent of ACP during apatite formation.^{36,37} As previous reported, one hypothesis is that the confinement provided by the membrane pore reduces the contact of the particles with the solution thus hindering redissolution and reprecipitation processes towards crystallization.³⁸ Actually, the study of the lifetime of

different amorphous minerals within limited volumes revealed several mechanisms by which confinement may affect the kinetics of precipitation, including slower transport of ions and reduced convection.^{39–41} In any case, the role of confinement is made clear by the presence of nanoscale heterogeneities from the outer to the inner surfaces of the tubes, some of the possible origin being the slower transport of ions, and preferential local dissolution and precipitation.

The stabilization of Sr(ACP) in confinement at Sr^{2+} content close to that found in the tissue of animals raises interesting questions concerning its impacts on bone biomineralization. Previous observations have pointed out that ACP infiltrates within the confines of the gap zone in collagen to form bone mineral.^{42,43} Notably, higher Sr^{2+} concentrations are found in young bone which is reported to be less crystalline as compared to the long term bone.^{44,45} On account of that, our results bring the notion that Sr^{2+} impacts on the early stages of bone mineralization as proposed for Zn^{2+} and non-collagenous proteins.^{46,47} Therefore, the role of Sr^{2+} on bone apatite may be further than a simple hetero-ionic exchange with Ca^{2+} or physical adsorption in the mature mineral, as usually described.⁴⁸ However, it is important to note that the mineralization *in vitro* is a simplified model of the much more complex biological systems, in which a multitude of specialized molecules and cells are involved in this process.

3.4. *In vitro* remineralization and Sr²⁺ release evaluation

Aiming at bone tissue regeneration, the bioactivity of the nanotubes was assessed *in vitro* by immersion in SBF, a solution which mimics the blood serum in terms of ionic composition and pH. This is a standard assay to give insights towards the bone forming ability of biomaterials *in vitro* under physiological conditions.²⁴

XRD revealed that the particles induced the precipitation of biomimetic apatite with typical broad diffraction peaks after 5 days of immersion in SBF (Figure 5b). It should be noted that SrCO₃ was formed in addition to apatite in the 50% Sr²⁺ sample, as indicated by the diffraction peaks at $2\theta = 25^\circ$ and 35° (indicated by S in the diffractogram). As discussed, this sample is likely formed by a mixture of Sr(ACP) and Sr(ACC). Therefore, in SBF these mineral phases undergo crystallization leading to the formation of apatite and SrCO₃. Interestingly, SrCO₃ is also a potential biomaterial to osteoporosis treatments, thus, the present experimental conditions and setup could constitute an alternative procedure to precipitate such mixed phases *in vitro*.^{33,49}

SEM images show that the nanotubes were covered by a newly rough layer formed by nanometric platelets confirming the deposition biomimetic apatite (Figure 5a). The fact that the morphology of the nanotubes was maintained, and their diameter increased after the exposure to SBF shows that they acted as template for the nucleation of the new phase, *i.e.* biomimetic apatite. This result also evidenced that the remineralization was spatially controlled, which is important to avoid pathological calcification *in vivo*. It is proposed that Ca²⁺ and PO₄³⁻ from SBF accumulate at the surface of templates leading to the formation of ACP which by redissolution and reprecipitation processes is converted into apatite, the most thermodynamically stable phase of CaP at physiological conditions.^{42,36} Then, apatite grows spontaneously by

1 consuming the templating particles and the ions from the medium including Na^+ , Mg^{2+}
2 and CO_3^{2-} , evolving towards a bone-like mineral in terms of morphology and
3 composition.⁵⁰ The incorporation of Sr^{2+} did not disturb the apatite-forming ability of
4 the tubes during the period of exposure investigated herein, in agreement with previous
5 findings.⁵¹ Hence, these results confirm the potential of these particles to induce
6 biomimetic remineralization in physiological environment.

7 The ability of the particles to release Sr^{2+} in the cell culture medium was
8 evaluated for 1, 3, 7, 14 and 21 days (Figure 5c). The amount of Sr^{2+} increased with the
9 time suggesting the sustained and controlled delivery for long periods. This is an
10 important result as most of the materials are reported to release the total amount of Sr^{2+}
11 within few days. It is noteworthy that the 50% Sr^{2+} sample released higher amount of
12 Sr^{2+} until the 14th day compared to the 100% Sr^{2+} sample. This is likely ascribed to the
13 amorphous nature of the 50% Sr^{2+} sample which may be more soluble than the highly
14 crystalline 100% Sr^{2+} sample. It is worth mentioning that SrCO_3 also contributes to the
15 sustained release of Sr^{2+} since in physiological environment this mineral is
16 progressively converted into biomimetic apatite by redissolution and reprecipitation as
17 previously reported.³³ After long periods, the concentrations found in the solutions fall
18 within the Sr^{2+} level in the serum of patients treated with strontium ranelate in which
19 bone healing was achieved in the range of 2–18 mg L^{-1} .²⁰ These results also show that
20 the composition of the particles can be tailored to release an appropriate level of Sr^{2+}
21 according to the application.

Figure 5. (a) SEM images and (b) X-ray diffraction patterns of the tubes containing different amounts of Sr²⁺ after 5 days of immersion into SBF. The diffractograms were indexed with the hydroxyapatite structure (JCPDS 9-432). The 50% Sr²⁺ sample led to the additional formation of SrCO₃ as observed by the additional peaks at $2\theta = 25^\circ$ and 35° (indicated by S in the diffractogram). (c) Sr²⁺ released from the 10% Sr²⁺, 50% Sr²⁺ and 100% Sr²⁺ tubes immersed in the cell culture medium at 37 °C for 12 hours, 1, 3, 7, 14 and 21 days. The amount of Sr²⁺ released by the particles falls in the therapeutic doses used for osteoporosis treatment.

3.5. *In vitro* assessment of cytocompatibility of Sr(CaP) nanotubes

It is well-known that an imbalance in the activity of osteoblasts and osteoclasts leads to bone diseases, in special osteoporosis. In this sense, the biological response of the particles was assessed *in vitro* by using pre-osteoblastic cells and osteoclasts differentiation protocols.⁵²

Confocal microscopy images obtained after 7 days of culture confirm that MC3T3-E1 cells reached confluence displaying a polygonal shape typical from osteoblasts (Figure 6a).⁵³ SEM images obtained after long term culture (21 days) show that cells remained attached to the substrate with flattened and elongated morphology characteristic of mature osteoblasts (Figure 6b). Moreover, the continuous monolayers of cells indicates that the particles did not disturb their adhesion and proliferation.⁵⁴ MTT assays obtained after 21 days of culture confirmed that the particles did not significantly influence MC3T3-E1 viability compared to the control, attesting to their negligibly toxicity towards osteoblasts at the concentration used here, even after long exposure periods (Figure 6c). It has been reported that concentrations of Sr^{2+} close to 17 mg L^{-1} (or higher) are needed to increase the osteoblasts proliferation which is higher than the range investigated herein and found in the plasma of humans treated with strontium ranelate ($\sim 10 \text{ mg L}^{-1}$).^{55,56} Still, it is important to emphasize that Sr^{2+} may accumulate in the bone tissue provoking pathological calcification.^{17,57} Therefore, low doses are preferred in Sr^{2+} -based therapies. In fact, osteoblasts viability is only the initial parameter to be evaluated during mineralization studies.

We further investigated the impacts of the particles on the ALP activity, an important marker of osteogenic activity and therefore bone formation.⁵⁸ After 7 days of culture the ALP activity was higher to 10% Sr^{2+} and 50% Sr^{2+} while there were no significative differences between 0% Sr^{2+} and 100% Sr^{2+} compared to the control

(Figure 6d). ALP activity is an early indicator of osteoblast phenotype differentiation, therefore the increased ALP activity on day 7 for cells cultured in the presence of the 10% and 50% Sr^{2+} particles suggests higher osteogenic differentiation as compared to the pure 0% and 100% Sr^{2+} particles. Likewise, a recent investigation reporting on the treatment of osteoblasts with a Sr^{2+} -flavonoid complex revealed that the increased ALP activity in the presence of Sr^{2+} can be associated to the overexpression of the enzyme whereas the cell viability was maintained.⁵⁹ Moreover, these results are in line with *in vivo* and *in vitro* studies showing that Ca^{2+} and Sr^{2+} mixed may led to an ideal microenvironment where these ions act synergistically to stimulate osteoblast metabolism at the early stages of differentiation.^{49,60} Indeed, the synergism between Sr and other elements such Fe and Cu in promoting bone mineral formation has been demonstrated.^{61–63} It is noteworthy that the replacement of Ca^{2+} by Sr^{2+} is reported to enhance the degradability of phosphates. In this regard, it has been speculated that Sr^{2+} substitution in apatite might additionally provide an ion-rich environment conducive for osteogenesis.^{60,64,66,67}

In general, the ALP activity increased after 14 days of culture, as already described for osteoblast-like cells.²⁶ This strengthens the particles ability to support MC3T3-E1 cells evolution towards mature bone-forming osteoblasts. Finally, Alizarin Red S assays revealed that the mineralization of extracellular matrix by osteoblasts was not negatively affected by the presence of the particles (Figure 6e and Figure S8). Although no statistical significance was found between the samples, a tendency of increased mineralization was observed for the 10% Sr^{2+} and 50% Sr^{2+} samples as compared to the control in agreement with ALP results. Overall, the activity of MC3T3-E1 cells was sustained in the presence of the particles strengthening their potential application as platform to induce positive osteoblast responses at low doses.

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1 Noteworthy, changes in the ζ -potential values after immersion in the cell culture
2 medium can be assigned to modifications on the particles' surface by adsorption of
3 serum proteins and other components which are known to enhance colloidal stability.⁶⁸

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6 **Figure 6.** (a) Confocal microscopy and (b) SEM images of MC3T3-E1 cells cultured for 7 and
7 21 days respectively, in the absence (control) and in the presence of Sr(CaP) nanotubes
8 containing 0% Sr²⁺, 10% Sr²⁺, 50% Sr²⁺ and 100% Sr²⁺. (c) Cell viability versus control
9 measured by MTT assay after 24 h, 7, 14 and 21 days of culture, (d) activity of ALP in the
10 osteoblasts' membrane fraction after 7 and 14 days of culture and (e) quantification of
11 mineralized nodules formed in the wells after 21 days of culture. Results represent the mean
12 values ± standard deviation for triplicate determination for each experiment. Multiple statistical
13 comparisons were performed by two-way ANOVA, **p < 0.01.

Next, we evaluated the impact of the particles on osteoclasts differentiation and function. Bone marrow-derived monocytes/macrophages (BMMs) were cultured under osteoclastogenic condition medium in the presence of the 0,10, 50 and 100% Sr^{2+} nanotubes and TRAP staining was performed to identify osteoclasts differentiation. The formation of multinucleated osteoclasts was inhibited by the Sr^{2+} loaded particles whereas no significant differences in relation to the control were found to the 0% Sr^{2+} particles (Figure 7a). Quantitative measurement confirmed that the number of TRAP positive cells was reduced as the Sr^{2+} concentration increased in the particles (Figure 7b). To evaluate the ability of the tubes to inhibit bone resorption, osteoclasts were cultured now on hydroxyapatite-coated plates and in the presence of the particles containing different amounts of Sr^{2+} . The formation of demineralization pits was prevented by the Sr^{2+} loaded particles (Figure 8a). Quantification of the areas of the pits confirmed that the demineralization capacity of osteoclasts was significantly reduced by the 10% Sr^{2+} particles or completely inhibited in the case of the 50% Sr^{2+} and 100% Sr^{2+} particles (Figure 8b).

Overall, these results are in agreement with previous investigations which demonstrated that Sr^{2+} released from biomaterials and particles inhibit osteoclast differentiation and resorption activities.⁶⁹ Nevertheless, Sr^{2+} levels much higher (88 mg L^{-1}) than those found in the serum of patients treated with strontium ranelate (10 mg L^{-1}) are usually described for eliciting such anti-osteoclastogenic effects.⁷⁰ Herein, a low amount of Sr^{2+} (less than 0.2 mg L^{-1}) was enough to significantly hinder osteoclast activity and differentiation. Interestingly, the % Sr^{2+} incorporated in these particles is in the physiological percentage found in bone tissue (10% mol with respect to Ca^{2+}).⁶³ Even the highest Sr^{2+} concentration used here (0.7 mg L^{-1}) was lower than that used in other investigations reporting on bioglasses able to delivery Sr^{2+} within the range

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described as efficient to trigger antiosteoporotic effects *in vivo* (~5 mg L⁻¹).¹⁹ This is an important feature, as the side effects of Sr²⁺ accumulation in bone cannot be overlooked; although high doses decrease differentiation and metabolism of osteoclasts it may also lead to pathological mineralization.^{17,17} Actually, the regulation of bone cells metabolism by Sr²⁺ is quite ambiguous. Several studies have demonstrated that osteoblasts response to Sr²⁺ might be a question of optimal environment rather than a concentration driven effect.^{49,62}

On the basis of our results and the literature presented in this discussion, we believe that tuning the Sr²⁺ and Ca²⁺ concentrations is crucial for the design of materials to bone regeneration and the approach described herein provides an effective way to do this.

Figure 7. (a) Representative images and (b) quantification of TRAP-positive osteoclasts cultured for 4 days. Bone marrow macrophages (BMMs) was cultured under M-CSF and RANKL stimulation, in the absence (control) and in the presence of the Sr(CaP) tubes containing 0% Sr^{2+} , 10% Sr^{2+} , 50% Sr^{2+} and 100% Sr^{2+} . Results represent the mean value $\pm$ standard deviation (n=5) representative of two independent experiments. One-way ANOVA, followed by Tukey's post-test. ****p< 0.001, compared to control. Scales bars represent 100 μm .

Figure 8. (a) Representative images and (b) quantification of resorption area induced by osteoclasts cultured in hydroxyapatite-coated plates for 5 days. Bone marrow macrophages (BMMs) was cultured under M-CSF and RANKL in hydroxyapatite-coated plates, in the absence (control) and in the presence of Sr(CaP) tubes containing 0% Sr²⁺, 10% Sr²⁺, 50% Sr²⁺ and 100% Sr²⁺. Results represent the mean value $\pm$ standard deviation (n=5) representative of two independent experiments. One-way ANOVA, followed by Tukey's post-test. ****p < 0.001, compared to control.

4. CONCLUSIONS

A bioinspired approach relying on physical confinement was developed to form Sr(CaP) nanotubes suitable to be used as building blocks of bone. In contrast to previous investigations, biomimetic platelets of apatite were formed here without the need of collagen, thus representing an innovative way to form fibril-like biomimetic biomaterials. The combination of Sr^{2+} and CaP yielded multifunctional particles able to induce osteoblasts proliferation, biomimetic remineralization and to reduce the differentiation and resorption activity of osteoclasts. Moreover, the increased ALP activity found for the 10% Sr^{2+} and 50 % Sr^{2+} samples supports their positive effect on the cell maturation and mineralization. Different from the current therapies, all these fundamental aspects for bone regeneration were triggered simultaneously here, and most importantly at low doses of Sr^{2+} which is crucial to avoid its side effects. By showing that Sr^{2+} can be effective at low concentrations when combined with CaPs, this investigation reconciliates the reports of pathological mineralization at excess of Sr^{2+} with its therapeutic effects. Going further, this investigation provides a facile strategy to produce a new generation of biomimetic ceramics with relevant biological responses.

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1 **5. ACKNOWLEDGMENTS**

2 C.B. Tovani thanks FAPESP (2014/24249-0) for the scholarships. A.P. Ramos
3 and P. Ciancaglini thank FAPESP for research grants (2017/08892-9 and 2016/21236-
4 0). We also thank Marcos A. E. Cruz and Lucas B. Nogueira for the support with cell
5 culture experiments. P. Ciancaglini and A.P. Ramos are CNPq researchers.

6
7 **6. ASSOCIATED CONTENT**

8 SEM images of the track-etched polycarbonate membrane; SEM images of
9 submicrometer tubes formed using 400 nm pore-size track-etched membranes;
10 Determination by TEM-EDX of the Sr²⁺ content in the particles formed in bulk and
11 confinement; Zeta potential (ζ) versus pH curves obtained for Sr(CaP) nanotubes; ATR-
12 FTIR spectra of Sr(CaP) nanotubes, STEM-HAADF image of 10% Sr²⁺ nanotube;
13 TEM-EDX mapping images of 10% Sr²⁺ and 50% Sr²⁺ nanotubes; STEM-EELS spectra
14 obtained at low temperature by LN₂ sample holder of 50% Sr²⁺ nanotube; Photographs
15 of Alizarin Red S stained osteoblasts' cultures.

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17 **7. REFERENCES**

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Figure 1. (a) Schematic representation of a track-etched membrane used as physical confinement to tune the morphology and size of the nanotubes. The side and top view of the membrane shows the cylindrical pores where the precipitation takes place. A SEM image of the surface of a membrane shows the pores. (b) Schematic representation of the precipitation of Sr(CaP) under either physical confinement (i, ii, iv) or in bulk solution (iii and v). The membranes were immersed into the PAA/salt solution for 12 h (i) and then placed in a desiccator containing $(\text{NH}_4)_2\text{CO}_3$ (ii) leading to the formation of nanotubes inside the cylindrical pores (iv). The particles (reddish SEM image) were isolated by dissolving the membranes with chloroform followed by centrifugation. A beaker containing the PAA/salt solutions was placed in the desiccator (iii) to conduct the precipitation from bulk solution (v) leading to agglomerated spherical particles (greenish SEM image).

204x167mm (150 x 150 DPI)

Figure 2. SEM images of the phosphate particles formed in bulk solution (a) and in confinement (b). XRD pattern of the particles synthesized in bulk solution (c) and in confinement (d). The products formed in bulk solution were indexed as follows: hydroxyapatite for 0% Sr²⁺ and 10% Sr²⁺ (JCPDS 9-432). The additional peaks (*) observed in the 0% Sr²⁺ sample may be due to OCP (JPDCS 26-1056). Strontianite (S) (JPDCS 01-071-4899) was found in the 50% Sr²⁺ sample in addition to an amorphous phase Sr(ACP). Strontium hydrogen phosphate was identified in the 100% Sr²⁺ sample (JCPDS 23-105026). The samples formed in confinement were identified as hydroxyapatite (0% Sr²⁺), Sr(ACP) (10% Sr²⁺ and 50% Sr²⁺) and strontium hydroxyapatite (100% Sr²⁺) (JPDCS 33-1348).

1064x875mm (120 x 120 DPI)

Figure 3. TEM-BF, STEM-HAADF images and SAED patterns of the tubes 0, 10, 50, and 100% Sr²⁺. STEM-HAADF images show that the 0% Sr²⁺, 10% Sr²⁺ and 50% Sr²⁺ tubes are hollow. The absence of crystalline reflections in the SAED patterns of the 10% Sr²⁺ and 50% Sr²⁺ tubes confirms their amorphous character depicted by XRD.

134x176mm (220 x 220 DPI)

Figure 4. (a,b) EELS spectra of the tubes containing 0%Sr²⁺, 10%Sr²⁺, 50%Sr²⁺ and 100%Sr²⁺. (c,d,e) EELS spectromicroscopy performed on the 50%Sr²⁺ tube showing the presence of several compositional heterogeneities.

242x188mm (150 x 150 DPI)

Figure 5. (a) SEM images and (b) X-ray diffraction patterns of the tubes containing different amounts of Sr²⁺ after 5 days of immersion into SBF. The diffractograms were indexed with the hydroxyapatite structure (JCPDS 9-432). The 50% Sr²⁺ sample led to the additional formation of SrCO₃ as observed by the additional peaks at 2θ = 25° and 35° (indicated by S in the diffractogram). (c) Sr²⁺ released from the 10% Sr²⁺, 50% Sr²⁺ and 100% Sr²⁺ tubes immersed in the cell culture medium at 37 °C for 12 hours, 1, 3, 7, 14 and 21 days. The amount of Sr²⁺ released by the particles falls in the therapeutic doses used for osteoporosis treatment.

169x96mm (220 x 220 DPI)

Figure 6. (a) Confocal microscopy and (b) SEM images of MC3T3-E1 cells cultured for 7 and 21 days respectively, in the absence (control) and in the presence of Sr(CaP) tubes containing 0% Sr²⁺, 10% Sr²⁺, 50% Sr²⁺ and 100% Sr²⁺. (c) Cell viability versus control measured by MTT assay after 24 h, 7, 14 and 21 days of culture, (d) activity of ALP in the osteoblasts' membrane fraction after 7 and 14 days of culture and (e) quantification of mineralized nodules formed in the wells after 21 days of culture. Results represent the mean values ± standard deviation for triplicate determination for each experiment. Multiple statistical comparisons were performed by two-way ANOVA, **p < 0.01.

149x112mm (220 x 220 DPI)

Figure 7. (a) Representative images and (b) quantification of TRAP-positive osteoclasts cultured for 4 days. Bone marrow macrophages (BMMs) was cultured under M-CSF and RANKL stimulation, in the absence (control) and in the presence of the Sr(CaP) tubes containing 0% Sr^{2+} , 10% Sr^{2+} , 50% Sr^{2+} and 100% Sr^{2+} . Results represent the mean value $\pm$ standard deviation ($n=5$) representative of two independent experiments. One-way ANOVA, followed by Tukey's post-test. **** $p < 0.001$, compared to control. Scales bars represent 100 μm .

210x152mm (120 x 120 DPI)

Figure 8. (a) Representative images and (b) quantification of resorption area induced by osteoclasts cultured in hydroxyapatite-coated plates for 5 days. Bone marrow macrophages (BMMs) was cultured under M-CSF and RANKL in hydroxyapatite-coated plates, in the absence (control) and in the presence of Sr(CaP) tubes containing 0% Sr²⁺, 10% Sr²⁺, 50% Sr²⁺ and 100% Sr²⁺. Results represent the mean value ± standard deviation (n=5) representative of two independent experiments. One-way ANOVA, followed by Tukey's post-test. ****p< 0.001, compared to control.

234x170mm (120 x 120 DPI)

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