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Camila Bussola Tovani, Thibaut Divoux, Sébastien Manneville, Thierry Azaïs, Guillaume P. Laurent, et al.. Strontium-driven physiological to pathological transition of bone-like architecture: A dose-dependent investigation. *Acta Biomaterialia*, 2023, 169, pp.579. <10.1016/j.actbio.2023.07.043>. <hal-04189351>

HAL Id: hal-04189351

<https://hal.sorbonne-universite.fr/hal-04189351v1>

Submitted on 31 Aug 2023

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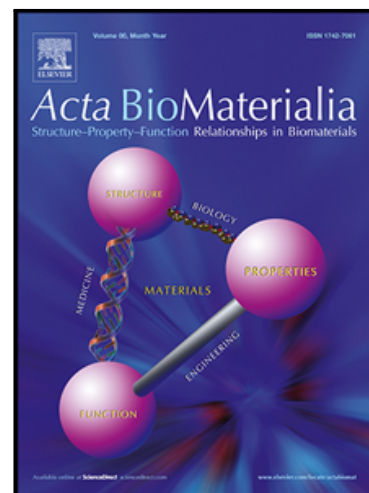


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PII: S1742-7061(23)00433-6
DOI: <https://doi.org/10.1016/j.actbio.2023.07.043>
Reference: ACTBIO 8865



To appear in: *Acta Biomaterialia*

Received date: 12 February 2023
Revised date: 19 June 2023
Accepted date: 24 July 2023

Please cite this article as: Camila Bussola Tovani , Thibaut Divoux , Sébastien Manneville , Thierry Azaïs , Guillaume Laurent , Marta de Frutos , Alexandre Gloter , Pietro Ciancaglini , Ana P. Ramos , Nadine Nassif , Strontium-driven Physiological to Pathological Transition of Bone-like Architecture: A Dose-dependent Investigation, *Acta Biomaterialia* (2023), doi: <https://doi.org/10.1016/j.actbio.2023.07.043>

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Strontium-driven Physiological to Pathological Transition of Bone-like Architecture: A Dose-dependent Investigation

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Abstract

Whilst strontium (Sr^{2+}) is widely investigated for treating osteoporosis, it is also related to mineralization disorders such as rickets and osteomalacia. In order to clarify the physiological and pathological effects of Sr^{2+} on bone biomineralization, we performed a dose-dependent investigation in bone components using a 3D scaffold that displays the hallmark features of bone tissue in terms of composition (osteoblast, collagen, carbonated apatite) and architecture (mineralized collagen fibrils hierarchically assembled into a twisted plywood geometry). As the level of Sr^{2+} is increased from physiological-like to excess, both the mineral and the collagen fibrils assembly are destabilized, leading to a drop in the Young modulus, with strong implications on pre-

osteoblastic cell proliferation. Furthermore, the microstructural and mechanical changes reported here correlate with that observed in bone-weakening disorders induced by Sr^{2+} accumulation, which may clarify the paradoxical effects of Sr^{2+} in bone mineralization. More generally, our results provide physicochemical insights into the possible effects of inorganic ions on the assembly of bone extracellular matrix and may contribute to the design of safer therapies for treating osteoporosis.

Keywords: bone, strontium, collagen, apatite, self-assembly

1. Introduction

Strontium (Sr^{2+}) is naturally present in the human body as a trace element in mineralized tissues (bones and teeth).[1] Over the past decades, the administration of Sr^{2+} has been described as beneficial as it can reestablish the imbalanced activity between osteoclasts (bone resorption cells) and osteoblasts (bone-forming cells), which is the primary cause of osteoporosis.[2] This has motivated the formulation of strontium ranelate, a drug with Sr^{2+} as the active component for treating and preventing osteoporosis, a major bone disease affecting hundreds of millions of elderly people worldwide.[3] Consequently, Sr^{2+} -based biomaterials and oral therapies have emerged over the last years as promising opportunities to treat this widespread bone disease. [4,5] However, paradoxically, clinical studies have reported a link between the high concentration of Sr^{2+} in bone and the development of disorders such as rickets and osteomalacia (also known as soft bones), which are characterized by abnormal mineralization of the extracellular matrix and accumulation of osteoid tissue.[6–8] The main clinical consequences of bone weakening caused by these disorders include skeletal deformities, poor growth, fractures, and severe pain.[9,10] This dichotomy poses clinical concerns for the main target of osteoporosis treatments (*e.g.*, elderly people and postmenopausal women), where an imbalance between Sr^{2+} uptake and removal is reported due to a decreased renal function.[11] In addition, Sr^{2+} is heterogeneously distributed in bones of osteoporotic patients treated with strontium ranelate with a selective uptake in newly formed osteon, which suggests the involvement of Sr^{2+} at the early stages of bone formation.[12,13]

In this context of biomineralization, *in vitro* models have shown that the structure of apatite, *e.g.*, the mineral phase of bone, is not altered at physiological-like concentrations of Sr^{2+} (*i.e.*, 10%),[12]

except for a slight increase in the lattice parameters.[14–17] Recently, we found that the bone-like apatite structure is disrupted in excess of Sr^{2+} , leading to the formation of a stable Sr^{2+} -rich amorphous calcium phosphate phase Sr(ACP).[17] Although the effects of Sr^{2+} have been widely investigated, the studies mainly focus on a physiological context as well as on the mineral component precluding the understanding at the higher level of bone hierarchy).[18] Therefore, there is a real benefit in studying the dose/effect relationship of Sr^{2+} with models that comprise the hybrid organic/inorganic structure of the tissue.

Here, we investigate physiological-like (10% Sr^{2+}) and excess accumulation-like (50% Sr^{2+}) amounts of Sr^{2+} in 3D biomimetic assemblies possessing the main structural hallmarks of bone tissue, *i.e.*, the plywood motif of collagen fibrils and carbonated apatite nanoparticles.[19] While 10% Sr^{2+} does not induce any structural modification, an excess of Sr^{2+} leads to the destabilization of both the fibrillar self-assembly of collagen and the apatite structure. Concomitantly, the local mechanical properties of the 3D scaffolds show a strong softening in the presence of an excess of Sr^{2+} , *i.e.*, the Young modulus drops by a factor of two compared to physiological-like conditions. In the pathological-like scenario, micrometric particles of Sr^{2+} -rich amorphous calcium phosphate Sr(ACP) are formed, destabilizing the typical three-dimensional architecture found in bone. We further show that such destabilization of the matrix microstructure leads to an impaired adhesion of preosteoblastic cells. Our observations correlate with the increased osteoid reported for Sr^{2+} -driven bone disorders[20–22] and contribute to clarifying the ambiguous role of this element on bone mineralization. Such results may have broad implications for the safer design of bone regenerative therapies. From a material perspective, this investigation highlights the intriguing destabilization of the collagen network by amorphous calcium phosphate particles, which are regarded as the precursor of bone minerals.[23][24]

2. Experimental procedure

2.1 Collagen extraction

Type I collagen monomers were extracted from rat tail tendons following a classical procedure. [25] Briefly, fresh tendons were washed with phosphate-buffered saline (PBS) solution to remove traces of cells and blood and then solubilized in 0.5 mol L⁻¹ acetic acid. The crude solution was centrifuged, and the supernatant was selectively precipitated with 0.7 mol L⁻¹ NaCl. Precipitated type I collagen was solubilized in 0.5 mol L⁻¹ acetic acid and desalted by dialysis against 0.5 mol L⁻¹ acetic acid. The

collagen concentration in the stock solution was determined by hydroxyproline titration [26] and then adjusted to 3 mg mL⁻¹.

2.2 Preparation of mineralized collagen matrices at different concentrations of Sr²⁺

Disk-shaped collagen matrices with a thickness of ~3 mm and a width of ~12 mm were prepared by combining continuous injection and reverse dialysis.[19] 15 mL of 1 mg mL⁻¹ collagen acidic solution was mixed with SrCl₂/CaCl₂/NaH₂PO₄/NaHCO₃ acidic solution (0.5 mol L⁻¹ acetic acid). Mixtures containing different Sr²⁺ molar percentages (namely 0, 10, and 50%) in relation to the total number of moles of divalent cations (Ca²⁺ + Sr²⁺) were prepared. Throughout the article, we refer to the samples prepared in the absence of Sr²⁺ as Coll-CaP-0%Sr²⁺ and those prepared in the presence of 10% Sr²⁺ and 50% Sr²⁺ as Coll-CaP-10%Sr²⁺ and Coll-CaP-50%Sr²⁺, respectively. The (Ca + Sr)/(P + C) ratio was kept constant and equal to 1.67, and the pH was ~3.5. The final concentrations of the salt precursors were CaCl₂ (46.1 mmol L⁻¹), SrCl₂ (46.1 mmol L⁻¹), NaH₂PO₄ (13.8 mmol L⁻¹), and NaHCO₃ (13.8 mmol L⁻¹). The collagen/salts mixtures were continuously injected in a closed dialysis mold. The bottom of the chamber was a dialysis membrane with a molecular weight cut-off of 12–14 kDa. A reverse dialysis process was performed against polyethylene glycol (PEG, 35 kDa, Fluka) dissolved in 5 mol L⁻¹ acetic acid up to 300 mg ml⁻¹. The ionic precursors of apatite were dissolved in both the collagen and the PEG solution. The global ionic strength (165.9 mmol L⁻¹) was kept constant throughout the process and was close to that described in physiological conditions. After complete collagen injection, dialysis was continued for 4 days in order to obtain the same concentration over the sample. Then, by increasing the pH in the range of 9 to 10 by NH₃ diffusion for 4 days, the precipitation of collagen fibrils and minerals was simultaneously triggered. Finally, the matrices were removed from the dialysis chamber and continuously washed with PBS (pH=7.4) in order to reach neutral pH. All experiments were carried out at a controlled temperature of 19 ± 2 °C in order to prevent collagen denaturation, and sterility conditions were applied throughout the procedure. The concentration of type I collagen in the matrices was assessed by hydroxyproline titration.[26] Control experiments were performed by precipitation of calcium/strontium phosphate particles in the absence of collagen. For this purpose, the mixtures containing different Sr²⁺ molar percentages (namely 0, 10, and 50%) in relation to the total number of moles of divalent cations (Ca²⁺ + Sr²⁺) were exposed to NH₃ vapor for 4 days. Throughout the article, we refer to the samples prepared in the absence of collagen as CaP-0%Sr²⁺, CaP-10%Sr²⁺, and CaP-50%Sr²⁺.

2.3 Bone sampling

2.3.1. Fresh bone

Bone was extracted (about 1 cm³) from the proximal part of the diaphysis and distal epiphysis of humerus and femur from 2-year-old healthy French sheep with a drill following the classical procedure described by Nuss *et al.*[27] The bone fragments were kept hydrated in phosphate buffer solution (0.01M PBS) until characterization (up to 1 hour) then directly prepared following the sample preparation for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) observations or directly inserted in a rotor for the solid-state nuclear magnetic resonance (ssNMR). The study was reviewed and approved by the IMM Recherche's Institutional Animal Care and Use Committee (IACUC) prior to the initiation of this study. The Animal Care and Use Committee of IMMR is registered at the CNREEA under the Ethics Committee n°37. The animal research center (IMM-Recherche) received an agreement (n°75-14-01) by the "Direction Départementale de la Protection des Populations". The studies were also performed in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources (published by the National Academy Press, Washington, D.C, 1996), as amended by the Animal Welfare Act of 1970 (P.L 91-579) and the 1976 amendments to the Animal Welfare were followed.

2.4 Sample characterization

2.4.1 Histological preparation for optical microscopy

Samples were fixed in 4% paraformaldehyde in PBS for 24 h, dehydrated through successive ethanol baths (v/v 70%-24h, 95%-3h, and 100%-3h) followed by 3 days bath in butan-1-ol and embedded in paraffin. Thick sections of 7 µm, transverse to the sample surface, were stained with picrosirius. This red staining is used to probe collagen in normal and pathological tissues.

2.4.2 Light microscopy under polarized light

Histological sections were observed with a Zeiss Axiomager A2 POL microscope equipped with crossed polarizers and an AxioCam CCD camera.

2.4.3 Scanning and transmission electron microscopy

For scanning electron microscopy (SEM) analysis, the matrices and bone sample were fixed in 3.6% glutaraldehyde in PBS (v/v) and dehydrated through successive ethanol baths (v/v 30%, 50%, 70%, 80%, 90%, 95%, and 100%) for supercritical CO₂ drying performed on a CPD 300 check. Samples coated with gold (10 nm layer) were studied by SEM using a Hitachi model S-3400N SEM at an accelerating voltage of 9 kV. For transmission electron microscopy (TEM) observations, the synthetic and biological samples were fixed with glutaraldehyde, dehydrated through successive ethanol baths (v/v 70%-24 h, 95%-3 h, and 100%-3 h), and embedded in Araldite. Ultrathin Araldite sections (60 or 70 nm) were contrasted with uranyl acetate and analyzed with a FEI TECMAI G2 Spirit Twin electron microscope operating at 120 kV.

2.4.5 Thermogravimetric analysis (TGA)

Experiments were performed on a thermo-microbalance instrument (TG 209 F1, Netzsch GmbH, Selb, Germany) with the air-dried sample. The measurement was performed from 20 °C to 800 °C in an air atmosphere with a heating rate of 10 °C min⁻¹. The final mass of the components was calculated by excluding the mass of free water. For this purpose, 100% weight of each sample was set at T = 120 °C.

2.4.6 Solid-state nuclear magnetic resonance (ssNMR)

Two dimensional ¹H-³¹P Cross Polarization Magic Angle Spinning (CPMAS) heteronuclear correlation experiments (¹H-³¹P HetCor) were performed on an AVANCE 300 Bruker spectrometer operating at $\nu(^1\text{H}) = 300.13$ MHz and $\nu(^{31}\text{P}) = 121.50$ MHz using contact time $t_{\text{CP}} = 1$ ms. The samples were packed in 4 mm zirconia rotors that were spun at a MAS frequency of 8 kHz. The chemical shifts were referenced to H₃PO₄ 85%wt for ³¹P (0 ppm) and adamantane for ¹H (0 ppm).

2.4.7 Nanoindentation for measuring local elastic properties

The local mechanical properties of mineralized collagen matrices were determined by nanoindentation at room temperature (22°C). Experiments were performed with a nanoindenter (PIUMA, Optics 11) equipped with a spherical probe made of glass (radius R=56.5 μm) and attached to a cantilever of calibrated stiffness (k=0.170 N/m). The test consisted of depth-controlled indentations of maximum depth set to 4 μm (yielding a typical contact radius between the sphere and the sample of about 10-15 μm) and performed over a square grid composed of 10x10=100

indentations, each spaced by 50 μm , hence covering a region of interest of size 500 μm x 500 μm . Indentations were performed following a symmetric displacement profile consisting of a loading phase (2 s), a constant force (so-called creep) phase (1 s), followed by unloading (2 s). Typical forces reached at the end of the loading phase were about 0.5 μN . Finally, for each indentation, the force-depth curve was fitted by a Hertzian contact model, yielding the effective Young modulus E_{eff} at the locus of the indent. The Young modulus E was then computed following $E = E_{eff}(1 - \nu^2)$, assuming a Poisson ratio ν of 0.5 for all samples.

2.4.8 Zeta potential (ζ) measurements

The surface charge of the scaffolds was investigated by ζ . The samples were prepared by cryogenic-grinding, and the resulting powders were dispersed in α -MEM supplemented with 10% fetal bovine serum. The measurements were carried out using a Malvern Zetasizer Nano ZS90.

2.5 Cell culture experiments

2.5.1 Cell viability –MTT (3-4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide)

assay

Osteoblastic lineage cells MC3T3-E1 (American Type Culture Collection-ATCCt) were cultured in a minimum essential medium (α -MEM, Gibco) supplemented with 10% fetal bovine serum and 1% (v/v) penicillin/streptomycin. The osteogenic medium was achieved by the addition of 50 mg/mL ascorbic acid and 10 mM beta-glycerophosphate. First, the collagen matrices were plated on the 24-well plates for 24 h to equilibrate with the culture medium. Then, the cells were seeded on the matrices at the density of 2×10^4 cells per well and incubated in air at 37 $^\circ\text{C}$ with 5% CO_2 . The culture medium was changed every two days. Cell viability was determined by the classic MTT assay after 7 and 14 days of culture using the protocol described by Mosmann.[28] Cell viability was expressed as the percentage of the average of three experiments as compared to the control (Ct) cultured on polystyrene wells for each day of culture (100%).

As a control, the cells were first seeded on 24-well plates at the density of 2.10^4 cells per well and incubated in air at 37 $^\circ\text{C}$ and 5% CO_2 with the CaP-0% Sr^{2+} , CaP-10% Sr^{2+} , and CaP-50% Sr^{2+} particles formed in the absence of collagen. For this purpose, the plated cells were allowed to attach to the

polystyrene well bottoms for 24h, followed by the replacement of the medium by a suspension of the calcium/strontium phosphate particles at $1\mu\text{g mL}^{-1}$ in the culture medium. The culture medium was changed every two days keeping constant the concentration of the particles. Cell viability was determined by MTT assay after 7 and 14 days of culture. Cell viability was expressed as the percentage of the average of three experiments as compared to the control (Ct) without treatment for each day of culture (100%).

2.5.2 Alkaline phosphatase (ALP) activity

ALP activity was determined by quantifying the hydrolysis of the substrate p- nitrophenyl phosphate (PNPP) in the plasma membrane fraction extracted from cells after 7 and 14 days of culture, according to the protocol described elsewhere.[29] The enzymatic activity was normalized by the total protein content which was estimated in the presence of 2% (w/v) sodium dodecyl sulfate using bovine serum albumin as standard.

2.5.3 Cell morphology

The cells cultured on the collagen matrices were fixed in 1.5% glutaraldehyde(v/v) at 4 °C for 12 h, dehydrated through a series of ethanol concentrations (v/v, 20, 50, 70, 80, 90, and 100%) then dried using supercritical CO₂ conditions and contrasted with osmium. After gold coating, the samples were observed on a Zeiss-EVO 50 SEM microscope under 20 kV accelerating voltage.

2.5.4. Statistical Analyses

For the studies of cell viability and ALP activity, multiple statistical comparisons were performed by one-way ANOVA in relation to the control, **p < 0.01. Results represent the mean values ± standard deviation for triplicate determination for one experiment.

3. Results and Discussion

Two types of scaffolds were prepared in the presence of 10% Sr²⁺ and 50% Sr²⁺ tending to mimic the two main scenarios described for Sr²⁺ incorporation in bone: (i) physiological (Coll-CaP-10%Sr²⁺ scaffold) and (ii) pathological (Coll-CaP-50%Sr²⁺ scaffold). In addition, control experiments were performed both by forming the bone-like scaffold in the absence of strontium (Coll-CaP-0%Sr²⁺ scaffold) and by precipitating mineral in the absence of collagen (CaP-0%Sr²⁺, CaP-10%Sr²⁺, and CaP-50%Sr²⁺). The different conditions are summarized in **Table a**.

3.1 The Impact of Sr²⁺ on Collagen Fibrils Mineralization

We first characterized the mineral phase formed in the collagen scaffolds at the hydrated state using ssNMR (**Figure 1**). From 2D ¹H-³¹P HetCor experiments, we observe that the Coll-CaP-10%Sr²⁺ scaffold exhibits a ³¹P NMR signature composed of two distinct resonances assigned to the coexistence of different domains in the mineral, which is the spectroscopic fingerprint of bone apatite[30] (Figure 1b *versus* Figure 1c): a crystalline core (sharp signal at $\delta(^{31}\text{P}) \approx 2.8$ ppm correlating with hydroxyl ions at $\delta(^1\text{H}) = 0$), and a disordered surface layer (broader signal at $\delta(^{31}\text{P}) \approx 3.2$ ppm correlating with water molecules at $\delta(^1\text{H}) = 4.8$ ppm). However, the spectrum for Coll-CaP-10%Sr²⁺ exhibits larger linewidths and a subtle shift compared to bone and Coll-CaP-0%Sr²⁺ (Figure S1, Supplementary Information) reflecting a higher local disorder in the crystalline core. This difference is ascribed to the replacement of Ca²⁺ by Sr²⁺ in the apatite structure, as previously described for both biomimetic apatite formed in the absence of collagen [16,17], and bone mineral.[14,31,32] In contrast, the ³¹P signal from the 2D ¹H-³¹P HetCor spectrum for Coll-CaP-50%Sr²⁺ is dominated by a broad linewidth together with a weak signal from apatite (Figure 1d). The similarity between the ³¹P projection of Coll-CaP-50%Sr²⁺ at $\delta(^1\text{H}) = 5.0$ ppm and the quantitative ³¹P spectra of CaP-50%Sr²⁺ show that the mineral formed in the scaffold is majoritarily Sr(ACP) (Figure 1e). This result reveals that the presence of collagen does not preclude the destabilization of apatite induced by high-dose of Sr²⁺.

After investigating the atomic environment of minerals, TEM observations were performed to characterize their morphology, size, and spatial distribution. Nanometric apatite platelets are formed at 10% Sr²⁺ (Figure 1f) as classically observed in bone (Figure 1g and Figure S2a) and in the biomimetic Coll-CaP-0%Sr²⁺ scaffold.[19,33] Overall, a comparison of the minerals shows that Sr²⁺ at a physiological-like concentration does not interfere with the ability of collagen to define the structure and morphology of apatite. In contrast, non-physiological dense rounded particles (~ 500 nm) are observed within the Coll-CaP-50%Sr²⁺ scaffold (Figure 1h and Figure S2b). In line with the NMR results, these particles resemble Sr(ACP) formed in the absence of collagen (Figure S3 , Supplementary Information).

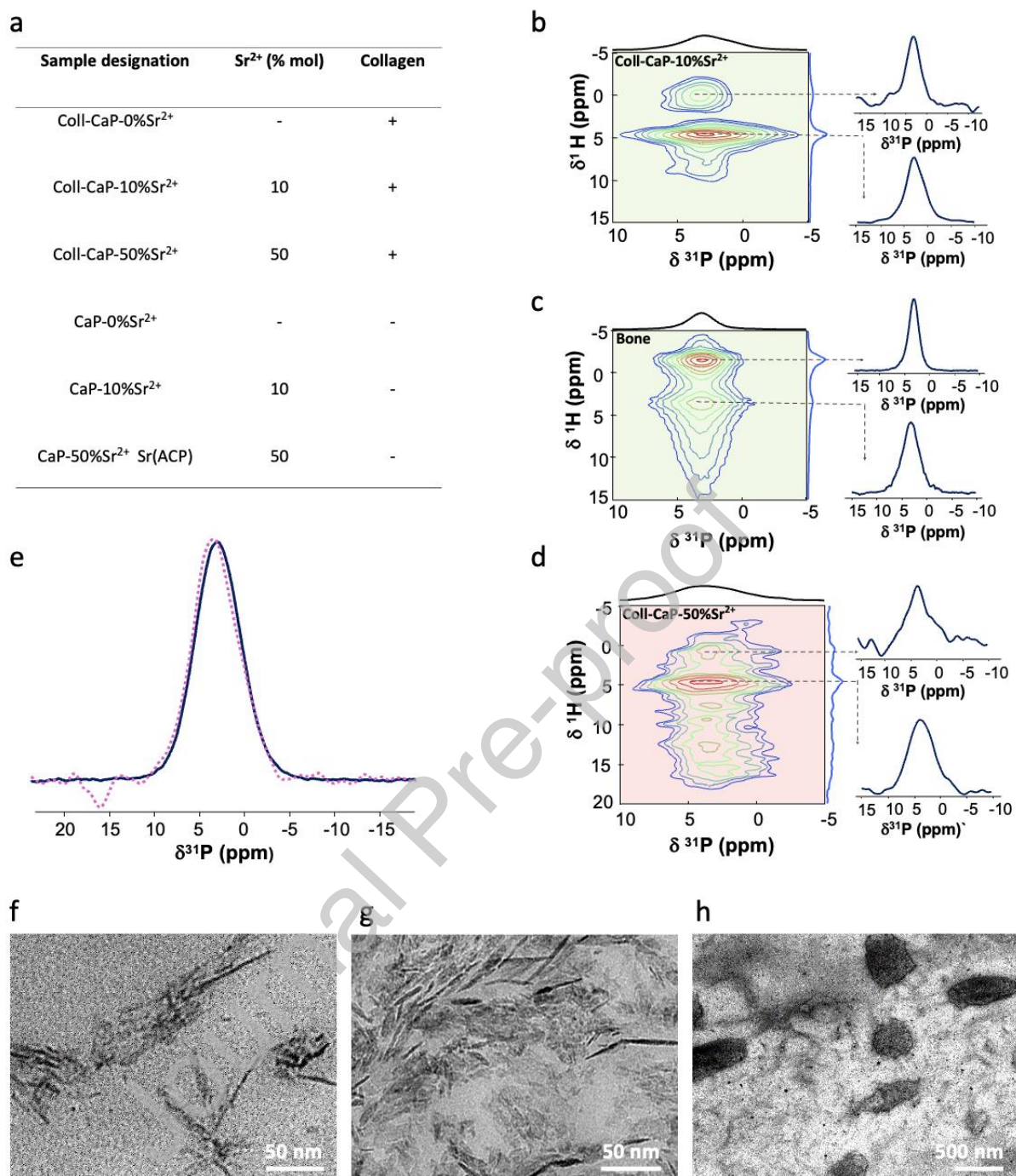


Figure 1. Effect of Sr²⁺ on bone-like apatite formed within collagen scaffold. Table (a). Summary of experimental conditions. ¹H-³¹P HetCor spectra of (b) Coll-CaP-10%Sr²⁺, (c) fresh bone and (d) Coll-CaP-50%Sr²⁺. (e) Comparison of the ¹H-³¹P HetCor extracted ³¹P row at $\delta(^1\text{H}) = 5.0$ ppm of Coll-CaP-50%Sr²⁺ scaffold and the ³¹P quantitative spectra of CaP-50%Sr²⁺ particles. TEM images of (f) Coll-CaP-10%Sr²⁺, (g) bone mineral, and (h) Coll-CaP-50%Sr²⁺.

3.2 The Impact of Sr^{2+} on the Bone-like suprafibrillar assembly of collagen (plywood pattern)

We next investigated how the formation of rounded Sr(ACP) *versus* Sr^{2+} -substituted apatite platelets influences the supramolecular assembly of collagen fibrils. It should be noted that previous studies have also investigated the mineralization of collagen fibrils in the presence of Sr^{2+} . [34,35] However, the impact of this element was never investigated on 3D biomimetic assemblies. To this aim, additional TEM investigations were performed on stained sections (**Figure 2**). Cross-striated fibrils are still observed at low concentrations of Sr^{2+} (Figure 2a, Coll-CaP-0% Sr^{2+} and Figure 2b Coll-CaP-10% Sr^{2+}) showing that Sr^{2+} does not affect the self-assembly and packing of collagen molecules. In contrast, no evident fibrillar organization was observed in the presence of Sr(ACP) (Figure 2c, Coll-CaP-50% Sr^{2+}), suggesting a destabilization of the collagen assembly at Sr^{2+} excess. To investigate whether this effect also occurs at larger scales, the scaffolds were then transversally fractured, and both the surface (Figure 2d-f) and the bulk (Figure 2g-i) fibrils were observed by SEM. Consistent with TEM images, it is also challenging to discriminate differences in the 3D architecture of collagen fibrils in Coll-CaP-0% Sr^{2+} and Coll-CaP-10% Sr^{2+} as both scaffolds display a tissue-like appearance with closely packed fibrils at the surface and a cholesteric twisted plywood geometry in bulk, similar to the pattern observed in bone [36] (Figure S4a and Figure S4b, respectively, Supplementary Information). Conversely, the Coll-CaP-50% Sr^{2+} scaffold displays a random fibril-like array at the surface and in bulk with the presence of macropores (arrows in Figure 2f) reflecting a higher interfibrillar space. The cholesteric geometry of collagen fibrils in Coll-CaP-0% Sr^{2+} (Figure 3a-a') and Coll-CaP-10% Sr^{2+} (Figure 3b-b') is further evidenced by polarized light microscopy images where a strong optical birefringence is observed throughout the thin section. After a 45° rotation of the polarizers, extinction of light occurs in areas where the collagen fibrils lie parallel to the direction of the polarizers indicating their cholesteric organization (Figure 3a' and Figure 3b'). Interestingly, Coll-CaP-50% Sr^{2+} also exhibits a nematic birefringent texture which indicates that isotropic and anisotropic domains exist within the scaffold. Nevertheless, cholesteric domains are never observed which also attests to the destabilization of collagen assembly at large scales (Figure 3c-c'). Overall, our observations fit into the two major landscapes of Sr^{2+} incorporation in bone: (i) the pathological accumulation, where disorder in the extracellular matrix is observed, and (ii) therapeutic administration, where no obvious changes are detected in the tissue microstructure.

Next, we determined the microscale mechanical properties of the different scaffolds with various contents in Sr^{2+} , in order to quantify the impact of the change in microstructure described above on the scaffold elasticity.

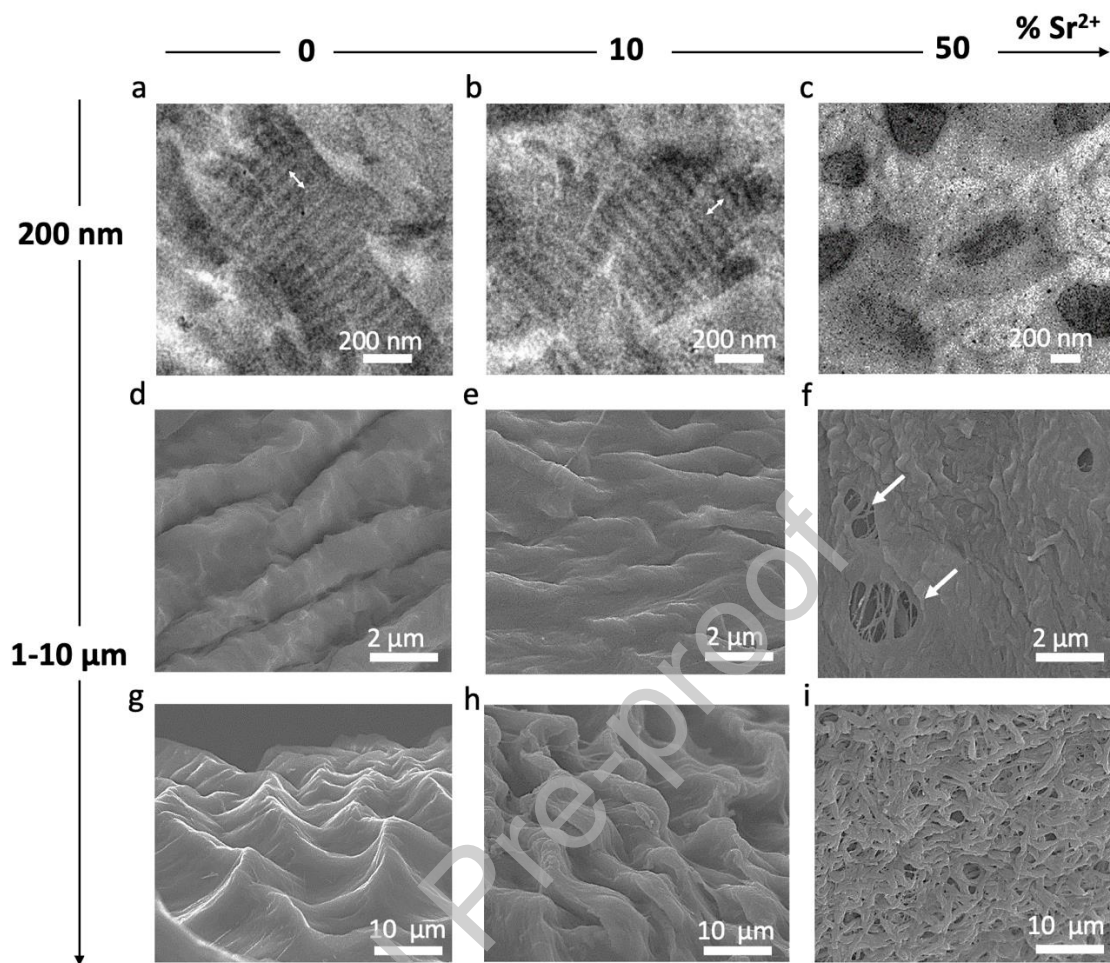


Figure 2. Effect of Sr^{2+} concentration on supramolecular assembly of collagen fibrils. TEM images of (a) Coll-CaP-0% Sr^{2+} , (b) Coll-CaP-10% Sr^{2+} , and (c) Coll-CaP-50% Sr^{2+} . Double headed arrows in a and b denote the 67 nm periodicity. SEM images showing the surface of (d) Coll-CaP-0% Sr^{2+} , (e) Coll-CaP-10% Sr^{2+} , and (f) Coll-CaP-50% Sr^{2+} . SEM images showing the interior of fractured (g) Coll-CaP-0% Sr^{2+} , (h) Coll-CaP-10% Sr^{2+} , and (i) Coll-CaP-50% Sr^{2+} . The white arrows in (f) indicate the presence of pores in the scaffold.

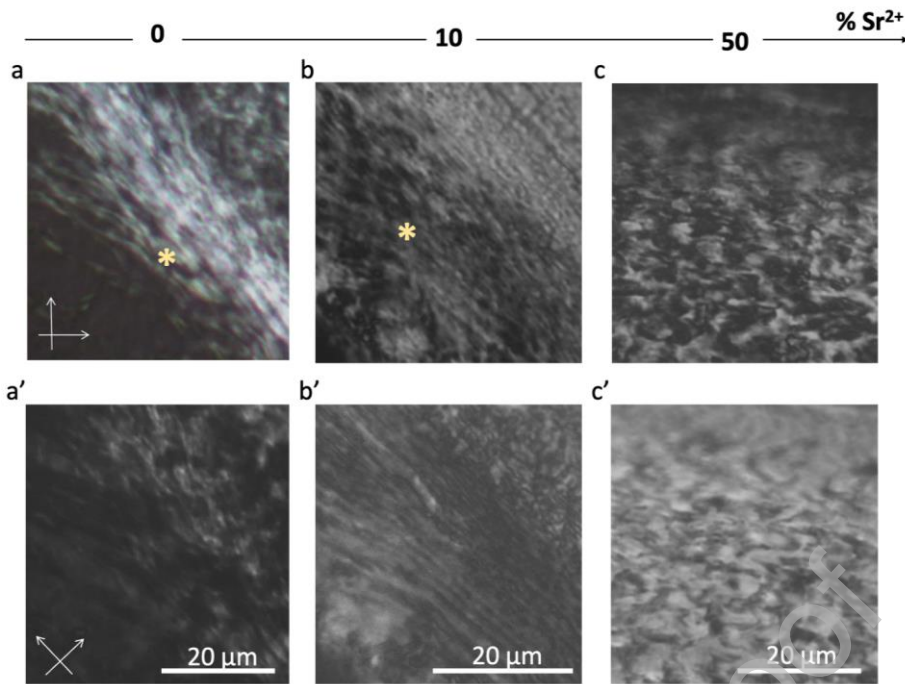


Figure 3. Effect of Sr^{2+} concentration on the organization of collagen scaffold. Polarized light microscopy images of (a-a') Coll-CaP-0% Sr^{2+} , (b-b') Coll-CaP-10% Sr^{2+} , and (c-c') Coll-CaP-50% Sr^{2+} . The asterisk in (a) and (b) indicate the presence of cholesteric domains that turn bright after a 45° rotation of both polarizers [see arrows in (a) and (a')].

3.3 Impact of Sr^{2+} on the local mechanical properties of collagen scaffold

Nanoindentation experiments allow measuring the local mechanical properties of the collagen scaffold on a region of interest corresponding to a square grid of size $500 \mu\text{m}$. Each indentation at the node of the grid provides a local elastic modulus averaged over a volume set by the contact area between the spherical indenter and the sample, which radius is about $10\text{-}15 \mu\text{m}$ for an indentation depth of $4 \mu\text{m}$. The size of the indentations was chosen to investigate the mechanical behavior of the scaffolds at the scale of one cell.

First, we observe that the Young moduli computed from the 100 indentations show a Poisson distribution for the two samples with the lowest content in Sr^{2+} , *i.e.*, Coll-CaP-0% Sr^{2+} and Coll-CaP-10%. In contrast, the Young moduli for Coll-CaP-50% Sr^{2+} follow a Gaussian distribution (Figure 4d). Moreover, samples Coll-CaP-0% Sr^{2+} and Coll-CaP-10% Sr^{2+} display comparable average values of about 8 kPa , whereas Coll-CaP-50% Sr^{2+} shows much weaker elastic properties characterized by an average Young modulus of about 4 kPa (Figure 4e). Such a drop in elasticity for the highest content in Sr^{2+} correlates well with the Sr^{2+} -induced destabilization of the collagen self-assembly and the presence of a porous-like microstructure in Coll-CaP-50% Sr^{2+} visible at the spatial scale of indentation.

Second, the indentation grid provides a spatially-resolved picture of the sample elastic properties in the region of interest, with a spatial resolution set by the distance between two consecutive indents, here fixed to 50 μm . Representative elasticity maps are reported in Figure 4a-c for the three types of collagen scaffolds. For samples Coll-CaP-0% Sr^{2+} and Coll-CaP-10% Sr^{2+} , we observe large-scale heterogeneities of size 200 μm , which might correspond to different arrangements of the fibrils at the sample surface. In contrast, Coll-CaP-50% Sr^{2+} displays a more homogeneous distribution of the Young modulus at the spatial scale investigated, insensitive to the presence of the non-physiological 500 nm particles. Such a discrepancy is reflected in the relative standard deviation, i.e., the ratio of the standard deviation to the mean value, of the distributions shown in Fig. 4d, which reaches 53% and 66% for Coll-CaP-0% Sr^{2+} and Coll-CaP-10% Sr^{2+} , respectively, while it is only of 31% for Coll-CaP-50% Sr^{2+} . Furthermore, a paired-sample *t*-test performed on the Coll-CaP-0% Sr^{2+} and Coll-CaP-10% Sr^{2+} data yield a *p*-value of 0.068 (which increases up to 0.127 when a single outlier at more than 5 times the mean value in the 10% Sr^{2+} data is disregarded), which is large enough to consider that these data are statistically equivalent with the standard significance level of 0.05. By contrast, unsurprisingly, the same *t*-test performed either on the Coll-CaP-0% Sr^{2+} and Coll-CaP-50% Sr^{2+} data or on the Coll-CaP-10% Sr^{2+} and Coll-CaP-50% Sr^{2+} data yield vanishingly small *p*-values ($<10^{-11}$). Thus, mechanical measurements show that collagen scaffolds in excess of Sr^{2+} are significantly softer, and more homogeneous than samples with a low Sr^{2+} content. Although simultaneous structural and mechanical measurements, e.g., through atomic force microscopy, would be needed to fully corroborate the impact of the fibril arrangement and/or of the presence of non-physiological particles on the local Young modulus, our *in vitro* results agree with the so-called “soft bones disorder” reported *in vivo*.^[10] Next, we investigated the impact of changes in the microstructural and mechanical properties of strontium-loaded scaffolds on bone forming cells.

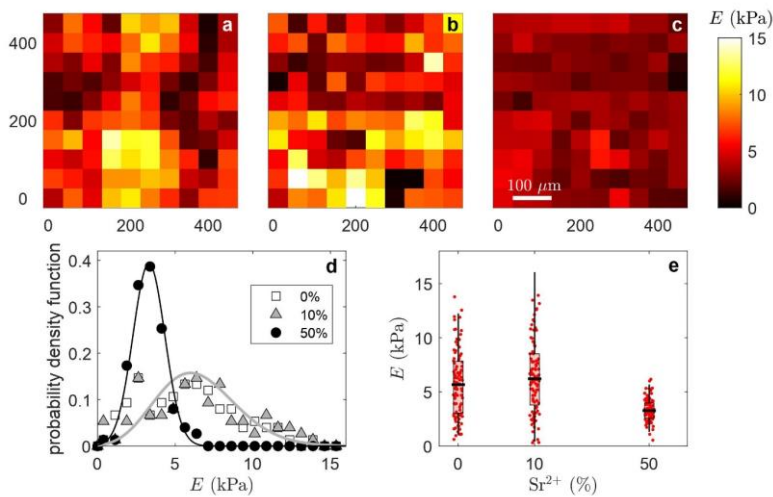


Figure 4. Effect of Sr^{2+} on the mechanical properties of collagen scaffold. Elasticity map over a region of interest of 500 μm square for (a) Coll-CaP-0% Sr^{2+} (b) Coll-CaP-10% Sr^{2+} , and (c) Coll-CaP-50% Sr^{2+} . Colors code for the Young modulus E . The probability density function of E is shown in (d) for the three samples of interest; the gray (resp. black) curve shows the best fit of the data by a Poisson (resp. Gaussian) distribution. The same data are reported as a Raincloud plot[37] in (d). For each data set, raw values of E determined at distinct locations of the sample appear as dots, while the box represents one standard deviation above and below the mean, itself marked by a horizontal black dash.

3.4 Bone cells response on strontium-loaded scaffolds

Pre-osteoblasts were cultured for 7 days on Coll-CaP-0% Sr^{2+} , Coll-CaP-10% Sr^{2+} , and Coll-CaP-50% Sr^{2+} scaffolds (**Figure 5a-c'**). Cells grew flat on Coll-CaP-0% Sr^{2+} (Figure 5a-a') and Coll-CaP-10% Sr^{2+} (Figure 5 b-b') displaying elongated morphology, which suggests their strong anchoring and spreading on these matrices.[38] The cell elongation indicates that the anisotropic matrices provided cues to guide their unidirectional adhesion along the axis of the collagen fibrils.[39,40] The suitable interaction between the cells and these substrates is further shown by the presence of filopodia (arrows in Figure 5a and Figure 5b) contacting neighboring cells and extending along the matrices surface.[41] In contrast, the cells seeded on Coll-CaP-50% Sr^{2+} display a spherical morphology, which reflects their low affinity for the scaffold (Figure 5c-c').[42] After 7 and 14 days of culture, the viability of cells was maintained on Coll-CaP-0% Sr^{2+} and Coll-CaP-10% Sr^{2+} and decreased on Coll-CaP-50% Sr^{2+} (Figure 5d), supporting SEM observations. The ALP activity, an early marker for osteoblasts differentiation, was also reduced for the cells cultured on Coll-CaP-50% Sr^{2+} suggesting that their evolution towards mature mineralizing osteoblasts was hindered as compared to the cells cultured on Coll-CaP-0% Sr^{2+} and Coll-CaP-10% Sr^{2+} scaffolds (Figure 5e).

The impact of different mineralization degrees of the scaffolds on cells behavior is discarded here since their mineral content is in the same range as demonstrated by TGA (20% for Coll-CaP-0%Sr²⁺, 18% for Coll-CaP-10%Sr²⁺ and 15% for Coll-CaP-50%Sr²⁺) (Figure S5, Supplementary Information). Furthermore, we can also exclude the influence of charge since the scaffolds display similar global charge in the cell culture environment with a zeta potential $\zeta \approx -6$ mV. Finally, the calcium phosphate particles formed without collagen (CaP-0%Sr²⁺, CaP-10%Sr²⁺ and CaP-50%Sr²⁺) did not display toxicity towards MC3T3-E1 (Figure 5f), which is in agreement with previous studies.[43] These results emphasize the impact of the scaffold structure/property on the cell response. Indeed, local defects in the bone matrix lead to impaired osteoblasts migration and maturation, preventing mineralization from progressing normally, as described in several bone disorders.[44,45] Noticeably, while it is commonly reported that high Sr²⁺ concentration impairs cell viability, [46] here we show the physicochemical interference of Sr²⁺ with the bone-like matrices comprising the destabilization of apatite and collagen network extends to cells behavior. It should be noted that although osteoblasts are responsible for setting the osteoid matrix which further densify and mineralize, bone remodeling process is not a fragmented sequence of events and, in fact osteoclasts and osteoprogenitors are intermingled on the eroded surfaces[47] Therefore, our model may help to improve our understanding on osteoblasts behavior during such orchestrated process.

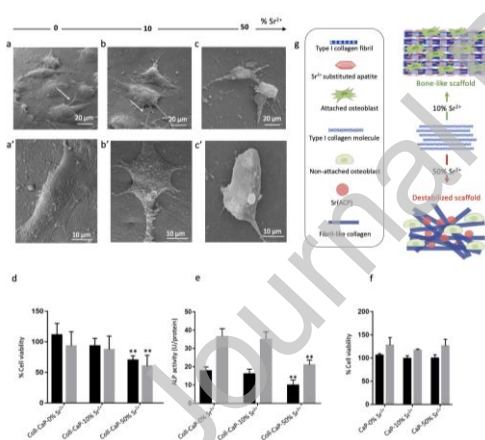


Figure 5. MC3T3-E1 pre-osteoblastic cells grown on collagen scaffolds or in the presence of CaP particles. (a-c and a'-c') SEM images of MC3T3-E1 cells cultured for 7 days on Coll-CaP-0%Sr²⁺, Coll-CaP-10%Sr²⁺, and Coll-CaP-50%Sr²⁺ scaffolds at two different magnifications. (d) % Cell viability versus control measured by MTT assay after 7 days (black bars) and 14 days (grey bars) of culture. (e) ALP activity of osteoblasts after 7 days (black bars) and 14 days (grey bars) of culture. (f) MC3T3-E1 cells grown in the presence of CaP-0%Sr²⁺, CaP-10%Sr²⁺ and CaP-50%Sr²⁺ particles. % Cell viability versus control measured by MTT assay after 7 days (black bars) and 14 days (grey bars) of culture. Results represent the mean values \pm standard deviation for triplicate determination for one experiment. Multiple statistical comparisons were performed by one-way ANOVA, **p < 0.01. (g) Schematic of the dose-dependent effect of strontium on bone components.

3.5 Mechanism of Sr²⁺-driven physiological to pathological transition of bone components

Based on the present results, we propose a possible mechanism for the Sr²⁺-driven structural destabilization of bone-like components in our model (Figure 5g). The excess of Sr²⁺ in the mineralizing medium hinders the crystallization of bone-like apatite platelets leading to the formation of micrometric Sr²⁺-rich amorphous calcium phosphate particles Sr(ACP) within the collagen scaffold. These non-biomimetic amorphous particles, in turn, disturb the collagen self-assembly both at the fibril and suprafibrillar scales found in bone, yielding weaker mechanical properties. Indeed, it is well-known that bone mineral platelets (nanometric dimension) decrease the distance between the molecules bringing a better cohesion to the bone material.[48,49] Conversely, it is possible that steric effects resulting from non-physiological microparticles preclude proper molecules/fibrils aggregation. Although Sr(ACP) was never reported *in vivo*, intracellular Sr²⁺-rich ACP was previously observed.[50–52] In addition, studies have shown that Sr²⁺ accumulates in the regions of new bone formation, more specifically near the mineralization front and in the region surrounding the osteoid, which may preclude bone maturation, as suggested by our model.[12] In our model, Sr²⁺ excess hinders the maturation of bone components, in agreement with the Sr²⁺-driven skeletal disorders.[6,21]

4. Conclusions

In summary, our *in vitro* biomimetic model shows that the structural hallmarks of bone tissue, namely collagen architecture, apatite structure, and cells activity, are destabilized at excess Sr²⁺. Concomitantly, no obvious alterations are produced at a physiological-like concentration of Sr²⁺. We propose that Sr(ACP) particles form within the scaffold at high doses of Sr²⁺, disturbing the architecture of collagen fibrils and leading to a decrease in the elasticity of the biomimetic scaffold. Along with *in vivo* observations, where a dose/effect relationship is observed for Sr²⁺, these findings account for the Sr²⁺ concentration-driven switch from physiological to pathological of the main components of bone tissue and therefore the paradoxical impacts of this element on bone mineralization. The destabilization of collagen architecture by Sr(ACP) is particularly intriguing since amorphous calcium phosphate is described as a crucial precursor in bone mineralization. It is worth mentioning that some reports have speculated that bone disorders such as fibrogenesis imperfecta may be associated with a large amount of amorphous calcium phosphate, which in turn interferes with the assembly of collagen fibrils.[31,32] Further investigation of the structure of organic and inorganic components in pathologic bone is needed to confirm whether the mechanism found in our

model is also observed *in vivo*. From a broader standpoint, our results shed new light on how pathologies destabilize the extracellular matrix of bone, while the versatility of our model opens perspectives for understanding the involvement of other ions and organic additives in bone mineralization in both physiological and pathological contexts.

Acknowledgments

C.B. Tovani thanks FAPESP (2014/24249-0) for the scholarships. A.P. Ramos and P. Ciancaglini thank FAPESP for research grants (2017/08892-9 and 2016/21236-0). We also thank Chakib Djediat and Corinne Illoul for their support with sample preparation for transmission electron and polarized light microscopies and Luc Behr from IMM for providing fresh ovine bone samples. P. Ciancaglini and A.P. Ramos are CNPq researchers.

Conflict of Interest

The authors declare no conflict of interest

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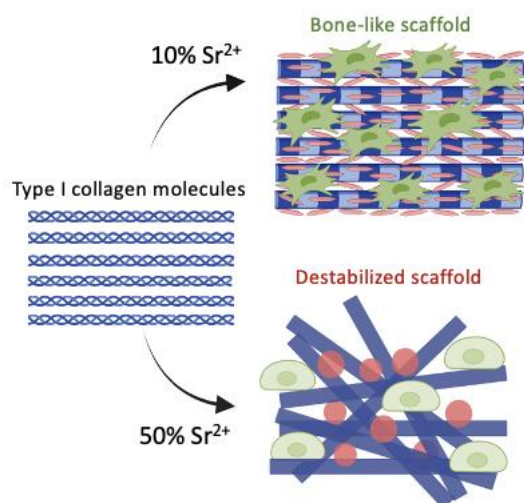
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Graphical abstract



Statement of Significance

Physiological-like (10% Sr²⁺) and excess accumulation-like (50% Sr²⁺) doses of Sr²⁺ are investigated in 3D biomimetic assemblies possessing the high degree of organization found in the extracellular of bone. Above the physiological dose, the organic and inorganic components of the bone-like scaffold are destabilized, resulting in impaired cellular activity, which correlates with bone-weakening disorders induced by Sr²⁺.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Journal Pre-proof