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Strontium-modification of porous scaffolds from mineralized collagen for potential use in bone defect therapy

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ABSTRACT

The present study describes the development and characterization of strontium(II)-modified biomimetic scaffolds based on mineralized collagen type I as potential biomaterial for the local treatment of defects in systemically impaired (e.g. osteoporotic) bone. In contrast to already described collagen/hydroxyapatite nanocomposites calcium was substituted with strontium to the extent of 25, 50, 75 and 100 mol% by substituting the $CaCl_2$-stock solution (0.1 M) with $SrCl_2$ (0.1 M) during the scaffold synthesis. Simultaneous fibrillation and mineralization of collagen led to the formation of collagen-mineral nanocomposites with mineral phases shifting from nanocrystalline hydroxyapatite (Sr0) over poorly crystalline Sr-rich phases towards a mixed mineral phase (Sr100), consisting of an amorphous strontium phosphate (identified as Collin's salt, $Sr_6H_3(PO_4)_5 * 2\ H_2O$, CS) and highly crystalline strontium hydroxyapatite ($Sr_5(PO_4)_3OH$, SrHA). The formed mineral phases were characterized by transmission electron microscopy (TEM) and RAMAN spectroscopy. All collagen/mineral nanocomposites with graded strontium content were processed to scaffolds exhibiting an interconnected porosity suitable for homogenous cell seeding in vitro. Strontium ions (Sr^{2+}) were released in a sustained manner from the modified scaffolds, with a clear correlation between the released Sr^{2+} concentration and the degree of Sr-substitution. The accumulated specific Sr^{2+} release over the course of 28 days reached 141.2 µg (~27 µg mg^{-1}) from Sr50 and 266.1 µg (~35 µg mg^{-1}) from Sr100, respectively. Under cell culture conditions this led to maximum Sr^{2+} concentrations of 0.41 mM (Sr50) and 0.73 mM (Sr100) measured on day 1, which declined to 0.08 mM and 0.16 mM, respectively, at day 28. Since Sr^{2+} concentrations in this range are known to have an osteo-anabolic effect, these scaffolds are promising biomaterials for the clinical treatment of defects in systemically impaired bone.

1. Introduction

Divalent strontium ions (Sr^{2+}) have been shown by several studies in the last years to have favorable influence on bone metabolism by effectively stimulating bone formation and inhibiting bone resorption, making strontium interesting for the treatment of osteoporosis [1–4]. Even though the exact mechanism of how strontium affects bone-related cells has not been fully understood yet, studies suggest an influence of Sr^{2+}-ions on the Wnt/β-catenin pathway [4], an interaction with the membrane-bound calcium sensing receptors [2,5,6], as well as an effect on osteoclast paracrine signaling [7,8] as possible cause for the bone-conserving effect of strontium treatment. These findings led to the clinical application of strontium in form of strontium ranelate as orally administered drug. Two clinical phase III trials with postmenopausal women evaluated the long-term efficacy and safety of strontium ranelate, showing that the daily application of 2 g strontium ranelate significantly reduced fracture risks and increased the bone mineral density [9,10]. However, the systemic application of strontium by oral

Abbreviations: CS, Collin's salt; HA, hydroxyapatite; hBMSC, human bone marrow-derived stromal cells; ICP-OES, inductively coupled plasma with optical emission spectrometry; MIP, maximum intensity projections; Nano-CT, nano-computed tomography; SEM, scanning electron microscopy; SrHA, strontium hydroxyapatite; TEM, transmission electron microscopy

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administration is afflicted with the drawback of a reduced bioavailability of only ~20% [11]. Moreover, due to safety concerns associated with the long-term application of strontium ranelate [12], current studies investigate organic strontium derivatives as safer alternative compounds to the already approved ranelate based carrier for Sr^{2+} ions e.g. the folic acid derivative anion (folate) [13,14]. Another approach to avoid systemic effects of Sr^{2+} ions is their local application: by integrating strontium into a biomaterial to be implanted into a bone defect the bioactive Sr^{2+} ions would be present directly at the specific defect site, which drastically reduces the necessary amount of drug and lowers the risk of possible side effects.

In bone tissue engineering, mimicking the extracellular matrix is a common strategy for the development of suitable scaffold matrices. Thereby, the scaffold material should constitute the physical microenvironment for cells allowing the colonization, proliferation and differentiation towards the desired tissue. A scaffold material fulfilling these characteristics is the well described nanocomposite mineralized collagen. Mineralized collagen is produced in a biomimetic process of synchronous collagen fibril reassembly and mineralization [15], leading to collagen with a tightly bound mineral phase of nanocrystalline hydroxyapatite (HA), which closely resembles the main extracellular matrix component of natural bone. By freeze drying and chemical crosslinking 3D scaffolds with an interconnecting porosity suitable for cell colonization can be fabricated [16]. Different in vitro studies demonstrated proliferation and osteogenic differentiation of human bone marrow-derived stromal cells (hBMSC) on mineralized collagen scaffolds [17–19]; scaffold resorption and new bone formation at the defect site have been demonstrated in vivo [20–22]. However, new tissue engineering concepts aim not only on “simple” biocompatibility of a biomaterial but also on an intrinsic potential to influence the host tissue in order to (locally) evoke a specific cellular response based on the cell–material interaction [23,24]. In osteoporotic bone the balance of bone remodelling, i.e. resorption by osteoclasts and formation by osteoblasts, respectively, is impaired. The higher resorption/bone formation ratio leads to the reduction of bone mass and altered microarchitecture and results in an increased fracture risk [25]. The integration of strontium into mineralized collagen scaffolds could locally increase bone formation due to the strontium's ability to simultaneously enhance osteoblast and inhibit osteoclast activity.

In this study, we demonstrate the development of Sr-modified mineralized collagen scaffolds, generated by substituting calcium with strontium during the scaffold synthesis to the extent of 25, 50, 75 and 100%. The Sr-modified scaffolds were analyzed by various methods (inductively coupled plasma with optical emission spectrometry (ICP-OES), Raman spectroscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), nano-computed tomography (Nano-CT), pycnometry, mechanical testing) to study the effect of Sr-substitution on scaffold composition and properties. The release kinetics of bioactive Sr^{2+} ions were determined to evaluate the applicability of Sr-modified mineralized collagen as potential biomaterial to fill defects, especially in osteoporotic bone.

2. Materials and methods

2.1. Scaffold preparation

Biomimetically mineralized collagen scaffolds without strontium substitution were prepared as described by Gelinsky et al. [16]. Based on this procedure strontium-containing mineralized collagen scaffolds were developed by partial or complete substitution of Ca^{2+} by Sr^{2+}. Briefly, pepsin digested collagen type I isolated from bovine tendon (kindly provided by Syntacoll, Germany) was dissolved in 10 mM hydrochloric acid. The collagen solution was then mixed with either 0.1 M $CaCl_2$ (for the Sr-free scaffolds) or with defined combinations of 0.1 M $CaCl_2$ and 0.1 M $SrCl_2$ solutions (for the Sr-modified scaffolds; Table 1). The pH was adjusted to 7.0 using 0.5 M TRIS (pH 11) and 0.5 M Sørensen phosphate buffer (pH 7.4), allowing the simultaneous collagen fibril reassembly and precipitation of HA and Sr-containing mineral phases at 37 °C for 12 h. The precipitate of mineralized collagen was collected by centrifugation, resuspended in distilled water (1.5 g mL^{-1}), filled into the cavities of a 96-well tissue culture plate, frozen at − 20 °C and freeze-dried subsequently. Chemical crosslinking of the obtained porous scaffolds (d = 6 mm, h = 3 mm) was conducted with 1 wt% EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide; Fluka, Germany) in 80 vol% ethanol for 1 h. Unbound carboxyl groups activated by EDC were quenched by incubation in 1 wt% aqueous glycine solution. After washing with distilled water the scaffolds were finally freeze-dried. For the Sr^{2+} release studies under sterile conditions the scaffolds were sterilized by γ-irradiation at 25 kGy.

Table 1
Nomenclature of prepared scaffold variants according to the applied degree of substitution of $CaCl_2$ (0.1 M) with $SrCl_2$ (0.1 M) during material synthesis.

Sample	$[Ca^{2+}]/[Sr^{2+}]$ ratio	
	Ca^{2+} [mol%]	Sr^{2+} [mol%]
Sr0	1	0
Sr25	0.75	0.25
Sr50	0.5	0.5
Sr75	0.25	0.75
Sr100	0	1

2.2. Elemental and phase analysis

2.2.1. Inductively coupled plasma with optical emission spectrometry

Inductively coupled plasma with optical emission spectrometry (ICP-OES, iCAP 6500 Duo View, Thermo Fisher Scientific, USA) was used for chemical element analysis of the mineral phase of the Sr-substituted scaffolds. Therefore, scaffolds (n = 3 of each group) were dissolved in 0.45 mL 65% HNO_3 (Merck, Germany) for 10 min at room temperature, while shaking at 400 min^{-1}. The digestion solution was then diluted with ultrapure water to 15 mL and filtered using 0.45 µm polypropylene (PP) syringe filter (VWR international, USA). The concentration of Ca^{2+}, Sr^{2+} and PO_4^{3-} was then determined after calibration measured from element standards (High Purity, USA) in bracketing mode.

2.2.2. Raman spectroscopy

To characterize the molecular composition of the generated scaffold variants Raman spectra were recorded using a spectrometer (Raman Rxn1, Kaiser Optical Systems Inc., USA) coupled to a light microscope (DM2500P, Leica Microsystems GmbH, Germany). For excitation, a 785 nm diode laser (Invictus 785 nm NIR, Kaiser Optical Systems Inc., USA) was used, propagated with a 100 µm optical fiber and focused by a 50 ×/0.75 microscope objective, leading to a focal spot of about 20 µm in diameter. The Raman scattering was collected in reflection configuration and sent to the spectrograph. Raman spectra were acquired in the range 600–3040 cm^{-1}. The spectral resolution was 4 cm^{-1}. An integration time of 2 s and 20 accumulations were used for spectra acquisition. The datasets were imported in Matlab (MathWorks Inc., USA). A baseline was calculated for each spectrum (function “msbackadj” of the Matlab Bioinformatics Toolbox) and the spectra were scaled to the same range by Vector normalization. Three spectra were recorded at different positions of each sample and averaged.

2.2.3. Loss on ignition

The amount of inorganic and organic fraction in the freeze-dried specimen was determined by measuring the loss on ignition. Therefore about 100 mg of the scaffold material were dried for 2 h at 120 °C with supply of air, weighed and transferred into a melting pot for pyrolysis in a muffle furnace (Padelttherm, Germany). After 1 h in air atmosphere