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Strontium eluting nanofibers augment stem cell osteogenesis for bone tissue regeneration

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ABSTRACT

Strontium is known to offer a therapeutic benefit to osteoporotic patients by promoting bone formation. Thus, toward engineering scaffolds for bone tissue regeneration we have prepared polymer nanocomposite scaffolds by electrospinning. Strontium carbonate nanoparticles ($nSrCO_3$) were added to poly(ε-caprolactone) (PCL) at 10 and 20 wt% to develop nanocomposite fibrous scaffolds (PCL/SrC10 and PCL/SrC20) with fiber diameter in the range of 300–500 nm. Incorporation of $nSrCO_3$ decreased crystallinity and the elastic modulus of PCL. The composite scaffolds released Sr^{2+} ions with up to 65 ppm in 4 days from the PCL/SrC20 scaffolds. Cell studies confirmed that the composite scaffold with 20% $nSrCO_3$ enhanced proliferation of human mesenchymal stem cells *in vitro*. There was marked increase in mineral deposition up to four folds in PCL/SrC20 suggesting enhanced osteogenesis. This was corroborated by increased mRNA and protein expression of various osteogenic markers such as BMP-2, Osterix and Runx2 in the PCL/SrC20 fibers. Thus, incorporation of $nSrCO_3$ in polymer scaffolds is a promising strategy for bone tissue engineering as an alternative to the use of labile growth factors to impart bioactivity to polymer scaffolds.

1. Introduction

Strontium, mainly absorbed through normal diet (2–4 mg/day) is found majorly as a trace element in the human body and constitutes about 0.00044% of body mass [1] and 0.035% of the overall calcium content [2]. Due to their similarity with calcium ions in the cellular transport pathways, strontium ions have a strong affinity for incorporation in the bone matrix during mineralization [1,3–5]. Interestingly, several *in vitro* studies [6,7] on strontium supplements and *in vivo* studies [8–10] on an osteoporotic drug (Strontium Ranelate) have suggested that strontium ions can facilitate bone formation. The underlying mechanism for these observations has been attributed to the ability of strontium ions to (i) stimulate differentiation and function of osteoblasts and (ii) inhibit bone resorption by preventing differentiation and activity of osteoclasts. Strontium thus plays an effective role in promoting osteogenesis and preventing resorption thereby minimizing bone loss *in vivo*. Owing to its multiple functions in influencing bone physiology, strontium offers a promising strategy to improve the bone guiding ability of biomaterials for repair and regeneration of the damaged and diseased tissues in the human body.

However, oral formulations of strontium containing drugs when prescribed at high dosages were reported to have several long term side effects [1,11]. Thus, novel strategies for targeted delivery of strontium ions to the defective bone site are being explored. Several studies have reported on the benefits of using strontium based biomaterials including strontium substituted bioactive ceramics like Bioglass [12], calcium phosphates [13,14] and strontium based coatings [15]. Furthermore, strontium substituted biomaterials were combined with polymers to prepare composite scaffolds [16,17], membranes [18] and hydrogels [19]. Thus, strontium inclusion enhances the bone forming ability of the biomaterials. It is further reported that the strontium has a significant role in inducing osteogenic differentiation of stem cells through different pathways such as Wnt [20,21] and Ras/MAPK [21,22], *etc.*

Surprisingly, till date, no near perfect bone guiding material has been clinically established despite the growing list of biomaterials. Whereas biodegradable polymers are well suited for the preparation of 3D scaffolds owing to their physico-chemical properties and processability, they lack bioactivity to induce bone formation. Biomolecules such as growth factors are not only expensive but labile and thus prone to loss in activity during processing when incorporated in the scaffold. In contrast, strontium salts offer a

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promising alternative for developing composite bone guiding scaffolds including carbonate [23–25], gluconate [26], and lactate [27] that are reported to be effective clinically. In this context, strontium carbonate ($SrCO_3$), also known as strontianite, has not been investigated for preparing composites for bone tissue scaffolds. $SrCO_3$ when administered at 600–700 mg/day orally as a source of strontium showed promising results in the treatment of osteoporosis [25]. In another recent study, a strontianite film developed on a sodium titanate surface enabled the local release of strontium ions *in vivo* from the implant surface, which in turn stimulated bone formation [28].

In this work, we report for the first time the preparation of composite nanofibrous scaffolds prepared by co-electrospinning of strontium carbonate nanoparticles ($nSrCO_3$) and polycaprolactone (PCL). PCL is a biodegradable polymer and has been widely used in clinically approved medical products and devices. Owing to its processability, PCL can be processed into a variety of 3D scaffolds for tissue engineering but lacks biochemical cues to promote osteogenesis. It has been reported that the nanofibrous architecture can induce stem cell osteogenesis even in the absence of biochemical cues through control of cell morphology [29]. We propose that the sustained release of strontium ions by incorporation of nanoparticles of $SrCO_3$ can further impart osteogenic property of PCL nanofibrous scaffolds. We studied the growth and osteogenic differentiation of human mesenchymal stem cells (hMSCs) on the composite scaffolds.

2. Experimental

2.1. Nanoparticle characterization

The as-received $SrCO_3$ nanoparticles (Reinste Nanoventures) were characterized using X-ray diffraction (XRD, XPERTPro, PANanalytical). To determine the size and morphology, the particles were dispersed in chloroform using a probe sonicator (Hielsher, Ultrasound technology) followed by drop casting on to a silicon wafer. The dried particles were imaged by scanning electron microscopy (SEM, Zeiss).

2.2. Preparation and characterization of nanofibrous scaffolds

2.2.1. Electrospinning

$SrCO_3$ nanoparticles particles were dispersed at 10% and 20% w/w of polymer solution of PCL ($M_n \sim 80000$, Sigma) in 3:1 ratio of chloroform and dimethylformamide (DMF) by bath sonication. The solid composition of the solution was fixed at 12% w/v. Attempts with variety of other solvents such as methanol, trifluoroethanol or hexafluoro-isopropanol or their combinations did not yield fibers. The nanofibrous scaffolds were prepared by electrospinning (Espin Nano). The samples hereafter are referred to as PCL, PCL/SrC10, PCL/SrC20 having $SrCO_3$ nanoparticle content of 0%, 10% and 20%, respectively. Electrospinning parameters (working distance, voltage applied, flow rate) varied across samples to obtain similar range of diameter are as follows: PCL (15 cm, 14 kV, 0.5 ml/h), PCL/SrC10 (15 cm, 12 kV, 0.5 ml/h) and PCL/SrC20 (12 cm, 10 kV, 0.5 ml/h). The fiber morphology was examined using SEM and fiber diameter was calculated by measuring the diameter of ten fibers each from three independent samples.

2.2.2. Thermal and mechanical analysis

Thermal analysis of the nanofibrous scaffolds was performed using differential scanning calorimetry (TA instruments). Second heating cycle of 0 °C to 100 °C at a constant rate of 10 °C/min was used for analysis. Percentage crystallinity (X_c) of samples was calculated as follows: $X_c = (\Delta H_c/\Delta H_0)^*100$, where ΔH_c is enthalpy of fusion of the sample and ΔH_0 is enthalpy of fusion of a fully crystalline material (ΔH_0 for PCL was taken to be 139.5 J/g) [30].

Mechanical characterization of the nanofibrous scaffolds (5 mm × 10 mm) was performed by dynamic mechanical analysis (DMA, TA Instruments) in a controlled force mode with a constant force of 3 N/min. The elastic modulus was taken as the slope of the linear region of the stress-strain plot. Three replicates were used for each sample and results are reported as mean ± S.D.

2.2.3. Strontium release

Release of Sr^{+2} ions from the scaffolds was measured using induction coupled plasma-optical emission spectroscopy (ICP-OES, Perkin Elmer). The fiber mat was cut to a size of 10 mm diameter to fit the wells of a 48 well plate. The mats in each well were dipped in 500 μl ultrapure water (Sartorius) for 4, 8, 12 days at 37 °C. The concentration was determined from the standard curve prepared using salt solutions of known concentration.

2.3. Biological studies

Primary human bone marrow-derived mesenchymal stem cells hMSCs (Stempeutics, Bangalore) from a 25 year old male donor were cultured in growth medium (GM) prepared from Knock-out DMEM (Invitrogen) supplemented with 15% MSC-qualified serum (Invitrogen), 1% antibiotic-antimycotic (Invitrogen) and 1% Glutamax (Invitrogen). Electrospun nanofibrous scaffolds were cut into circular discs (10 mm diameter) to fit in the wells of 48 well plate. Prior to cell seeding, the scaffolds were sterilized under UV for 1 h. 2500 cells were seeded on each scaffold and the medium was refreshed every four days.

2.3.1. Cell attachment and proliferation on scaffolds

Cellular attachment was measured 1 day after cell seeding and cell proliferation was assessed at day 7 and day 14 by measuring the cellular DNA content using the Picogreen dsDNA quantification kit (Invitrogen) as reported recently [31]. To examine the morphology, cells were fixed in 3.7% formaldehyde for 15 min, permeabilized with 0.2% TritonX-100 for 5 min and incubated in Alexa Fluor 546 (Invitrogen) for 15 min followed by DAPI (Invitrogen) for 1 min to stain F-actin and nuclei, respectively. Samples were imaged using an inverted *epi*-fluorescence microscope (Olympus IX-71).

2.3.2. Mineralization

hMSC mineralization on scaffolds *in vitro* was studied at day 14 and 21 by staining the fixed scaffolds with 2% Alizarin Red S (Sigma) dye for 30 min. The samples were washed with water until the excess dye was removed. The visual assessment was carried out by collecting the images of stained scaffolds and mineral quantification was done spectroscopically at 405 nm after extracting the bound dye by incubating the samples in 5% SDS and 0.5 N HCL for 30 min. The data are presented after normalizing to DNA content (measure of cell numbers).

2.3.3. mRNA and protein expression of osteogenic genes

Gene expression was assessed by quantitative mRNA detection of Osterix, Runx-2, BMP-2 and Osteopontin genes by real time PCR (RT-PCR) with GAPDH as the internal house-keeping gene. The primer sequences are listed in Table 1. Total RNA was extracted from scaffolds at 21 days using RNeasy kit (Qiagen). 1 μg of DNase treated RNA was used for the first strand cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed using 10 ng of cDNA in a 10 μl PCR reaction (1X SYBR mix, Kappa) and primers with the ABI PRISM 7500 (Applied Biosystems). The PCR reactions were performed as follows: 95 °C for 60 s, 40 cycles at 95 °C for 15 s and 60 °C for 15 s. Data were computed by $2^{-\Delta\Delta CT}$ method [32].