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Murine osteoblastic and osteoclastic differentiation on strontium releasing hydroxyapatite forming cements

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ABSTRACT

Ionic substitutions in hydroxyapatite (HA) scaffolds and self-setting cements containing Sr^{2+} ions incorporated are particularly of interest in bone regeneration. To date, the approach widely used to incorporate Sr^{2+} ions into HA cements has been the addition of Sr^{2+} containing salts, such as $SrCO_3$, $SrCl_2 \cdot 6H_2O$, or $SrHPO_4$. However, this approach is dependent upon the relative solubility of Sr^{2+} containing salts with respect to calcium phosphate (CaP) precursors. Therefore, in the current study Sr^{2+} substituted dicalcium phosphate dihydrate (DCPD) was first synthesized and directly reacted with tetracalcium phosphate (TTCP) to form Sr^{2+} substituted HA forming cements. Rietveld refinement indicated that after one week of aging in phosphate buffered saline, cements prepared with and without Sr^{2+} were composed of 75% HA and 25% unreacted TTCP by weight. Cements prepared with 10% Sr^{2+} DCPD exhibited increased compressive strengths in comparison to unsubstituted cements. Increased MC3T3-E1 proliferation and differentiation were also observed on the cements prepared with increasing Sr^{2+} content. It was concluded that both the scaffold microstructure and Sr^{2+} ion release supported osteogenic differentiation. With respect to osteoclastic differentiation, no statistically significant differences in TRAP activity or cell morphology were observed. This suggests that the amount of Sr^{2+} released may have been too low to influence osteoclast formation in comparison to unsubstituted cements. The results obtained herein demonstrate that the use of Sr^{2+} substituted DCPD precursors rather than individually separate Sr^{2+} containing salts may be a useful approach to prepare Sr^{2+} containing HA cements.

1. Introduction

Sr^{2+} substituted calcium phosphates (CaPs) have shown promise in the development of synthetic bone graft substitutes [1,2]. This is primarily due to the capability of Sr^{2+} ions to stimulate the differentiation of osteoblast progenitor cells, limit osteoclastic resorption, and enhance the radiopacity of CaPs [3]. To improve the clinical application of Sr^{2+} substituted CaP based scaffolds, self-setting Sr^{2+} substituted hydroxyapatite (HA) and dicalcium phosphate dihydrate (DCPD) forming cements have been developed [4]. HA forming CaP cements are traditionally of greater interest due to their less acidic setting reaction and improved mechanical properties. They have also been prepared using a wide range of CaP precursors. However, they are most commonly prepared either by the hydrolysis of α-tricalcium phosphate (α-TCP) or by an acid–base reaction between tetracalcium phosphate (TTCP) and either dicalcium phosphate anhydrous (DCPA) or DCPD [5,6].

To date, the majority of work reported on Sr^{2+} substituted HA forming cements has been with formulations prepared using α-TCP rather than TTCP and either DCPA or DCPD. Schumacher et al. for instance prepared Sr^{2+} releasing HA forming cements using α-TCP and DCPA with mixtures of $CaCO_3$ and $SrCO_3$ forming either Sr^{2+} substituted HA or clusters of $SrCO_3$ within a HA matrix [7,8]. The capability of these cements to support the proliferation and differentiation of human mesenchymal stem cells was also demonstrated. $SrCl_2 \cdot 6H_2O$ has also been used to introduce Sr^{2+} ions to HA forming cements prepared using α-TCP [9,10]. In both cases, the incorporation of Sr^{2+} into the HA crystal structure, which is necessary for the sustained release of Sr^{2+} ions, is dependent upon the relative solubility of the Sr^{2+} containing precursors with respect to α-TCP and the participation of Sr^{2+} in the re-precipitation process. Therefore, the synthesis of Sr^{2+} containing CaP precursors and their direct use in the cement forming setting reaction to form the HA cements, may be a more efficient and prudent route to incorporate Sr^{2+} ions into the resultant HA crystal structure during the cement setting reaction rather than using Sr^{2+}

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Table 1
The amounts (expressed as 10^{-3} mol) of Ca^{2+}, Sr^{2+}, and PO_4^{3-} containing precursors used in the synthesis of dicalcium phosphate dihydrate (DCPD).

	$CaCl_2 \cdot 2H_2O$	$SrCl_2 \cdot 6H_2O$	Na_2HPO_4
Undoped	50	–	50
5% Sr	47.5	2.5	50
10% Sr	45	5	50

containing salts. This approach has previously been explored using Sr^{2+} substituted α-TCP [11].

In one of the few reports where Sr^{2+} substituted HA forming cements were prepared using TTCP and either DCPA or DCPD rather than α-TCP, Guo et al. prepared $SrHPO_4$ (DSP) by precipitation and studied the cements formed using ternary mixtures of TTCP, DCPA, and DSP [4]. As a result of the varying solubility and particle sizes of DSP and DCPA, unreacted DSP was detected in the cements formed after up to one week in 100% humidity conditions. Despite this, further work studying these cements indicated improved in vitro cytocompatibility and in vivo degradation when implanted intramuscularly and in a femoral defect in rabbits with increasing Sr^{2+} content [12]. However, the incorporation of Sr^{2+} ions into the HA crystal structure is once again dependent upon the relative solubility of the two acidic precursors namely, DCPA and DSP.

Therefore, in the current study Sr^{2+} substituted DCPD (SrDCPD) of varying levels of substitution was directly prepared for the first time to the best of what is reported in the literature by a simple precipitation route under aqueous conditions and reacted with TTCP to form Sr^{2+} substituted HA (SrHA) forming cements. It was further hypothesized that the use of SrDCPD would lead to the incorporation of Sr^{2+} into the HA formed during the cement setting reaction in a very controlled manner during the acid–base reaction between TTCP and DCPD rather than involving a competing reaction of the two CaP precursors with a Sr containing salt as reported in the literature. The influence of varying the Sr^{2+} content in the cement scaffolds generated by this simple chemical reaction approach involving substituted precursors on the setting kinetics, phase composition, and compressive strength was characterized. In addition, the capability of Sr^{2+} containing cements to support the in vitro osteoblastic and osteoclastic differentiation of mouse preosteoblasts as well as monocytes was also studied and reported herein.

2. Materials and methods

2.1. DCPD precipitation

All the reagents used for the synthesis were acquired from Sigma-Aldrich and used in the form in which they were received. DCPD was precipitated under aqueous conditions using a method previously described [13]. $CaCl_2 \cdot 2H_2O$ and $SrCl_2 \cdot 6H_2O$ mixtures (Table 1) were dissolved in 100 mL of deionized water. This solution was added dropwise to 100 mL of a 0.5 M Na_2HPO_4 solution under constant stirring at room temperature. The precipitate formed was collected ten minutes after combining the solutions allowing the reaction illustrated in Eq. (1) to proceed to completion. The precipitate formed was subsequently centrifuged and washed using DI water. The final washing step was performed with ethanol and the precipitate was dried at room temperature under vacuum to avoid the formation of DCPA.

$$(1-a)CaCl_2 + aSrCl_2 + Na_2HPO_4 \rightarrow Ca_{(1-a)}Sr_aHPO_4 \bullet 2H_2O + 2NaCl. \quad (1)$$

2.2. Powder characterization

The phase composition of the powders formed after precipitation was determined using X-ray diffraction. Accordingly, X-ray diffraction patterns were collected using a Philips X-Pert PRO diffractometer employing Cu Kα radiation (λ = 1.5406 Å) with a Si-detector (X'celerator). The X-ray generator was operated at 45 kV and 40 mA at a 2θ range of 10–70° with a step size of 0.0167° and a time per step of 3 s. Elemental analysis of the precipitated powders was performed using inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP duo 6500, Thermo Scientific). Briefly, 10 mg of powder samples were dissolved in 3.5% HNO_3. These solutions were further diluted and analyzed using ICP-OES employing known standards for Ca^{2+}, Sr^{2+}, and PO_4^{3-}. Standard solutions have been prepared using the 3.5% HNO_3 stock solution. Scanning electron microscopy (SEM, Philips XL30 FEG ESEM) was used to analyze the particle size and microstructure of the powders formed after precipitation. Samples were sputter coated with palladium (Cressington, 108) prior to SEM analysis.

2.3. Cement characterization

Powder mixtures of 45% DCPD containing varying amounts of Sr substitution and 55% TTCP (<53 μm, HIMED, Old Bethpage, NY), by weight, were prepared using a mortar and pestle. The cement pastes were formed by combining TTCP–DCPD powder mixtures with either DI water or a 1.25% Na_2HPO_4 solution using a powder to liquid ratio of 2.2 g mL^{-1}. The resulting pastes were filled into 10 mm diameter cylindrical molds and allowed to fully set at 37 °C. The cement reaction between TTCP and DCPD resulting in the formation of HA is illustrated by Eq. (2).

$$Ca_4(PO_4)_2O + CaHPO_4 \bullet 2H_2O \rightarrow Ca_5(PO_4)_3OH + 2H_2O. \quad (2)$$

The initial and final setting times were determined using a Gillmore Needle Apparatus (ASTM C-266). After complete setting, the cements were immersed in phosphate buffered saline (PBS, Lonza, Allendale, NJ) and incubated at 37 °C for up to one week. At various time points during the one week period, samples were collected and analyzed using X-ray diffraction. Rietveld refinement was used to quantitatively analyze the phase composition (HighScore Plus, Version 3.0e) using the ICSD reference patterns for HA (22060), DCPD (16132), and TTCP (2631). The wet compressive strengths of the cement scaffolds after immersion in PBS was analyzed using a 2 kN load cell (Instron, Norwood, MA) and a cross head speed of 1.3 mm min^{-1}. A minimum of six repeats were performed for each condition at each time point using 6 mm diameter cylindrical scaffolds which were 12 mm long. The % porosity of the

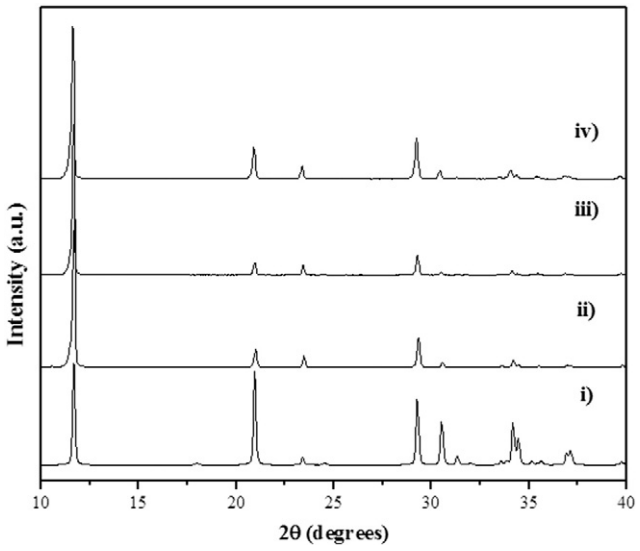

Fig. 1. The X-ray diffraction patterns of i) DCPD (JCPDS 09-0077), ii) unsubstituted DCPD, iii) 5% Sr DCPD, and iv) 10% Sr DCPD after drying overnight under vacuum.