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Study of hMSC proliferation and differentiation on Mg and Mg–Sr containing biphasic β-tricalcium phosphate and amorphous calcium phosphate ceramics

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ABSTRACT

Biphasic mixtures of either Mg^{2+} or combined Mg^{2+} and Sr^{2+} cation substituted β-tricalcium phosphate (β-TCP) and amorphous calcium phosphate (ACP) were prepared using a low temperature chemical phosphatizing and hydrolysis reaction approach. Scaffolds prepared using the cation substituted calcium phosphates were capable of supporting similar levels of human mesenchymal stem cell proliferation in comparison to commercially available β-TCP. The concentrations of Mg^{2+}, Sr^{2+}, and PO_4^{3-} released from these scaffolds were also within the ranges desired from previous reports to support both hMSC proliferation and osteogenic differentiation. Interestingly, hMSCs cultured directly on scaffolds prepared with only Mg^{2+} substituted β-TCP were capable of supporting statistically significantly increased alkaline phosphatase activity, osteopontin, and osteoprotegerin expression in comparison to all compositions containing both Mg^{2+} and Sr^{2+}, and commercially available β-TCP. hMSCs cultured in the presence of scaffold extracts also exhibited similar trends in the expression of osteogenic markers as was observed during direct culture. Therefore, it was concluded that the enhanced differentiation observed was due to the release of bioactive ions rather than the surface microstructure. The role of these ions on transforming growth factor-β and bone morphogenic protein signaling was also evaluated using a PCR array. It was concluded that the release of these ions may support enhanced differentiation through SMAD dependent TGF-β and BMP signaling.

1. Introduction

Calcium phosphates (CaPs) have been widely studied as bioactive components in synthetic bone graft substitutes [1]. Scaffolds prepared using CaP phases such as, dicalcium phosphate dihydrate (DCPD) and β-tricalcium phosphate (β-TCP), have gained increasing interest in hard tissue regeneration due to their resorbability under physiological conditions [2]. Furthermore, the release Ca^{2+} and PO_4^{3-} ions upon degradation may enhance the osteogenic differentiation of both stem and progenitor cells towards mature osteoblasts [3]. In addition to their application as bioactive components in bone graft substitutes, CaPs have also been studied in the form of coatings and as drug delivery vehicles [4–6]. Due to their wide range of applications, the controlled manipulation of both the physical and chemical properties of CaPs, such as particle size, solubility, roughness, and crystallinity have become of increasing importance to improve the performance of CaP based medical devices.

Ionic substitutions have previously been explored to provide controlled manipulation of the physicochemical properties of CaPs. For example, work by Enderle et al. has shown that the cationic substitution of Ca^{2+} by Mg^{2+} in β-TCP stabilizes β-TCP at temperatures > 1100 °C, enabling increased sintering and densification [7]. Ionic substitutions may also enhance the biocompatibility of CaP based scaffolds, since mineralized tissues are known to contain small amounts of various elements in addition to Ca and P, such as Mg and Sr, which are believed to play a critical role in regulating both structure and function [8]. Enhanced Mg^{2+} substitution in CaPs is known to stabilize amorphous calcium phosphates (ACPs) which, along with octacalcium phosphate (OCP), is believed to be a precursor to the formation of hydroxyapatite (HA) during mineralization in vivo [9,10]. Interestingly, although the presence of Mg^{2+} in biological apatite has been shown to decrease throughout the calcification process, the exact role of Mg^{2+} in the regulation of bone repair is yet to be determined [8,11].

In addition to Mg^{2+}, Sr^{2+} substituted CaPs have also gained significant interest due to the capability of Sr^{2+} to support osteoblastic differentiation while simultaneously inhibiting osteoclastogenesis

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[12]. Work by Yang et al. showed that enhanced in-vitro osteogenic differentiation was observed in human mesenchymal stem cells (hMSCs) cultured in the presence of increased Sr^{2+} concentrations through Wnt/β-catenin signaling. Sr^{2+} substituted HA-collagen composites were also implanted into rat calvarial defects and Sr^{2+} substituted HA was shown to significantly enhance in-vivo bone regeneration in comparison to HA [13]. However, increased Sr^{2+} concentrations may lead to rickets and renal failure [14]. Mg^{2+}-Sr^{2+} co-substituted CaPs have also been explored to further mimic native mineralized tissues [15]. However, the majority of studies performed to date have focused on the synthesis and characterization of these materials rather than evaluating either their in-vitro or in-vivo biocompatibility.

In the current study, the co-substitution of Mg^{2+} and Sr^{2+} in β-TCP was studied by hydrolyzing DCPD precipitated with Mg^{2+} and Sr^{2+}. The synthesis of Mg^{2+} substituted β-TCP (β-TCMP) from Mg^{2+} substituted DCPD using this approach was previously explored [16]. Upon increasing the Mg^{2+} concentration, increased amorphous content was formed in addition to β-TCMP. Interestingly, β-TCMP prepared with a 50% Mg/(Ca + Mg) ratio using this hydrolysis approach also supported significantly increased levels of osteogenic differentiation in a mouse preosteoblast cell line in comparison to scaffolds prepared using commercially available β-TCP [17]. However, the roles of the chemical and physical cues provided by the scaffold were not determined and the mechanism through which these scaffolds supported differentiation was also not studied. In the present study therefore, the influence of Mg^{2+} and Sr^{2+} co-substitution on the phase composition, physicochemical properties, proliferation, and mechanisms through which these scaffolds support the hMSC osteogenic differentiation has been reported.

2. Materials and methods

2.1. Synthesis of Mg and Mg-Sr substituted β-TCP

$CaCl_2 \cdot 2H_2O$ (ACS Reagent ≥ 99.0%, Acros Organics), $MgCl_2 \cdot 6H_2O$ (ACS Reagent, ≥99.0%, Acros Organics), and $SrCl_2 \cdot 6H_2O$ (ACS Reagent, ≥99.0%, Sigma Aldrich) were dissolved in 100 ml of deionized water (Table 1). This solution was then slowly added under constant stirring to 100 ml of 0.5 M of disodium hydrogen phosphate (Na_2HPO_4, ≥99.0% Sigma-Aldrich) at room temperature. The mixture was stirred for 15 min prior to centrifuging and washing the precipitate formed with deionized water. In all cases, the (Ca + Mg + Sr)/P mole ratio was kept constant at a value of 1. The washed precipitate was then dispersed in 200 ml of deionized water and was boiled with reflux for 2 h. After refluxing, the powders were once again collected by centrifugation and washed with deionized water prior to drying overnight at 60 °C.

2.2. Characterization of Mg and Mg-Sr substituted β-TCP

X-ray diffraction (XRD) was performed 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. The specific surface area of the particles formed was determined using the multi point Brunauer Emmett Teller (BET, ASAP 2020, Micromeritics) technique. The true density was measured using helium pycnometry (Accupyc II 1340, Micromeritics). Thermogravimetric and differential thermal analyses were performed using a Netzsch STA 409 PC DTA/TGA. These measurements were performed by heating the samples in air to 1000 °C at a rate of 10 °C min^{-1}. Heat treatments of the as synthesized powder and pressed pellets were performed using a Lindberg box furnace (Lindberg/blue, Riverside MI). Elemental analysis of the as prepared powders was performed using inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP duo 6500, Thermo Scientific). Scanning electron microscopy (SEM, Philips, XL30) and high resolution transmission electron microscopy (HRTEM, JEOL JEM-2100F with Gatan GIF-Tridiem) were also used to observe the particle morphology and crystallinity.

Table 1
The amounts by weight of Ca, Mg, and Sr containing precursors used in the precipitation of Mg and Mg-Sr doped $CaHPO_4 \cdot 2H_2O$.

Mg-Sr	$CaCl_2 \cdot 2H_2O$	$MgCl_2 \cdot 6H_2O$	$SrCl_2 \cdot 6H_2O$
35–15%	3.68	3.56	2.00
40–10%	3.68	4.07	1.33
45–5%	3.68	4.57	0.67
50%	3.68	5.08	–

2.3. Human mesenchymal stem cell maintenance and culture

Human mesenchymal stem cells (hMSCs) obtained from the normal human bone marrow were purchased from Lonza (Lonza, Allendale, NJ) and were cultured under 37 °C, 5% CO_2, and 95% relative humidity. The cells collected after the third passage were used in all experiments and were cultured in growth media containing minimum essential media α (MEMα) supplemented with 20% FBS and 1% P/S. Osteogenic differentiation was induced after 7 days of culture using the growth media supplemented with 100 nM dexamethasone, 50 μM ascorbic acid, and 10 mM β-glycerophosphate.

2.4. MTT cell viability

13 mm diameter discs of the as prepared powders were formed by uniaxial pressing (Carver, Wabash, IN) of 0.35 g of powders with an applied load of 2500 psi followed by heat treatment to 600 °C for 4 h. The cell viability of hMSCs seeded directly on the surface of these disc shaped scaffolds was assessed using the MTT assay (Vybrant MTT Cell Proliferation Assay Kit, Invitrogen, Carlsbad, CA). Water soluble MTT is reduced to insoluble formazan crystals in the living cells. Formazan is then solubilized and its concentration can be determined by measuring optical density at 570 nm. 1 ml of growth media containing 12 mM MTT was added to each well and incubated for 4 h. A 0.1 g/ml solution of sodium dodecyl sulfate in 0.01 M hydrochloric acid was then added and the samples were incubated for an additional 15 h. Absorbance was then measured at 570 nm.

2.5. Live/dead staining

At the various time points of interest, the samples were washed with PBS and were then incubated for 30 min with calcein AM and ethidium homodimer-1 diluted in PBS (Invitrogen, Live/dead Staining Kit) at room temperature. After incubation, the samples were once again gently washed with PBS prior to imaging using fluorescence (Olympus CKX41).

2.6. Alkaline phosphatase assay

hMSCs were lysed using a lysis buffer following the manufacturer's protocol (CelLytic M, Sigma Aldrich). 170 μl of 1 g L^{-1} p-nitrophenyl phosphate (pNPP) dissolved in 0.2 M tris buffer (SIGMAFAST™ p-Nitrophenyl phosphate tablets, Sigma Aldrich) was added to 30 μl of cell lysate. The samples were then incubated at 37 °C for 1 h. The reaction was stopped by the addition of 20 μl of 0.3 N NaOH. The concentration of p-nitrophenyl (pNP) in solution after incubation was determined by measuring the absorbance at 405 nm. pNP standards were prepared by diluting pNP in 0.02 N NaOH. Alkaline phosphatase (ALP) activity was also normalized with respect to total protein concentration.