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Magnesium substitution in the structure of orthopedic nanoparticles: A comparison between amorphous magnesium phosphates, calcium magnesium phosphates, and hydroxyapatites

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ABSTRACT

As biocompatible materials, magnesium phosphates have received a lot of attention for orthopedic applications. During the last decade multiple studies have shown advantages for magnesium phosphate such as lack of cytotoxicity, biocompatibility, strong mechanical properties, and high biodegradability. The present study investigates the role of Mg^{+2} and Ca^{+2} ions in the structure of magnesium phosphate and calcium phosphate nanoparticles. To directly compare the effect of Mg^{+2} and Ca^{+2} ions on structure of nanoparticles and their biological behavior, three groups of nanoparticles including amorphous magnesium phosphates (AMPs) which release Mg^{+2}, calcium magnesium phosphates (CMPs) which release Mg^{+2} and Ca^{+2}, and hydroxyapatites (HAs) which release Ca^{+2} were studied. SEM, TEM, XRD, and FTIR were used to evaluate the morphology, crystallinity, and chemical properties of the particles. AMP particles were homogeneous nanospheres, whereas CMPs were combinations of heterogeneous nanorods and nanospheres, and HAs which contained heterogeneous nanosphere particles. Cell compatibility was monitored in all groups to determine the cytotoxicity effect of particles on studied MC3T3-E1 preosteoblasts. AMPs showed significantly higher attachment rate than the HAs after 1 day and both AMPs and CMPs showed significantly higher proliferation rate when compared to HAs after 7 days. Gene expression level of osteoblastic markers ALP, COL I, OCN, OPN, RUNX2 were monitored and they were normalized to GAPDH housekeeping gene. Beta actin expression level was monitored as the second housekeeping gene to confirm the accuracy of results. In general, AMPs and CMPs showed higher expression level of osteoblastic genes after 7 days which can further confirm the stimulating role of Mg^{+2} and Ca^{+2} ions in increasing the proliferation rate, differentiation, and mineralization of MC3T3-E1 preosteoblasts.

1. Introduction

Over the last few years magnesium phosphates have gained some attention, albeit a lot less than they deserve, for their potential applications in the biomedical field specifically in orthopedics. To appreciate the prospects of magnesium phosphates in such applications, an understanding of the physiological roles of magnesium in the human body is essential. It is well known that magnesium ions are the fourth most abundant cations in mammals after sodium, potassium, and calcium [1,2]. Also, magnesium is the second most prevalent intracellular cation [1,2]. Inside mammalian cells magnesium plays multiple essential roles including: regulation of calcium and sodium ion channels, stabilizing DNA, being a cofactor and catalyzer for many enzymes, and stimulating cell growth and proliferation [2,3].

There are three major reasons that justify the application of magnesium phosphates in orthopedics: 1) magnesium can easily substitute for calcium in body minerals due to their chemical similarities [4]; 2) presence of Mg^{2+} ions in bone minerals and body fluid can influence the bone mineral metabolism, formation and crystallization processes [5,6]; and 3) magnesium phosphates have higher dissolution rate than calcium phosphates [7,8].

During the last several decades, reports on investigations on calcium phosphates with orthopedic applications have been numerous, as opposed to a handful of reports on magnesium phosphates [9,10]. Magnesium phosphates are components of minerals such as kidney stone and bone, however most of magnesium related studies have been on orthopedic cements [9,10].

There is limited work studying the interactions between magnesium phosphates and bone cells. Tamimi et al. reported that newberyite ($MgHPO_4 \cdot 3H_2O$) and cattiite ($Mg_3(PO_4)_2 \cdot 22H_2O$) demonstrated biocompatibility with osteoblast cultures and induced osteoblast adhesion and differentiation [11]. Ewald et al. showed that osteoblast activity was

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Table 1
Compositions of reaction solutions.

DI water		$NaNO_3$	$Ca(NO_3)_2 \cdot 4H_2O$	$Mg(NO_3)_2 \cdot 6H_2O$	$NaHCO_3$	$MgCl_2 \cdot 6H_2O$	KH_2PO_4	HNO_3
AMP	10 ml	–	–	–	0.6803 g	0.62325 g	0.4083 g	–
CMP	10 ml	5 g	1 g	0.91 g	–	–	0.345	0.5 ml

higher on brushite ($CaHPO_4 \cdot 2H_2O$) and struvite ($NH_4MgPO_4 \cdot 6H_2O$) cements than the calcium deficient hydroxyapatite ($Ca_9(PO_4)_5HPO_4OH$) cements when cell activity was normalized to cell number [12]. They showed that the highest survival rate and cell activity belonged to osteoblasts cultured on magnesium phosphate, struvite-based cements [12]. Expression levels of osteoblastic specific proteins on calcium and magnesium phosphate surfaces were determined. Collagen (COL), osteopontin (OPN), bone sialoprotein (BSP), alkaline phosphatase (ALP), and ostecalcein (OCN) expression levels were monitored, and all except COL showed higher expression levels in brushite than other groups [12]. Osteoblasts cultured on struvite showed higher osteoblastic characteristics when compared to their counterparts seeded on polystyrene [12]. However, this study did not include calcium–magnesium phosphate biomaterials and their impact on osteoblastic markers expression levels.

Calcium magnesium phosphates (CMPs) are mostly referred to as magnesium doped calcium phosphates. Products of Mg^{2+} ion substitution into calcium phosphates can be either in the amorphous phase or in the crystal structures which can cause a series of changes to the biological and physical properties of hosts. The presence of magnesium ions is believed to retard the nucleation and growth of hydroxyapatite (HA) in biological mineralization process *via* blocking of active growth sites through adsorption of Mg^{2+} ions at the crystal surface [6,13–15]. In the chemical synthesis of Mg^{2+} substituted HA using aqueous solution, Mg^{2+} substitution for Ca^{2+} in the structure of HA occurs only over a limited composition range (up to about 10 at.%) [16,17]. It has been reported that Mg^{2+} substitution for Ca^{2+} causes a reduction in the lattice parameters of HA [18]. Moreover, the degree of crystallinity of Mg-substituted HA decreases with increasing Mg^{2+} content [16,17]. Mg-doped HA displays increased solubility with respect to stoichiometric HA, which may be related to reduced crystallinity and/or increased surface hydration [18,19].

Previously, our group used a microwave assisted technique to synthesize amorphous magnesium phosphate (AMP) in a nanospherical form from an aqueous solution containing Mg^{2+} and HPO_4^{2-}/PO_4^{3-} [20]. The as-prepared AMP was shown to assist the proliferation of preosteoblasts [20]. Our group demonstrated that sustained release of magnesium and phosphate ions incorporated into the AMP and polylactic acid (PLA) structure could stimulate a series of cell responses. Magnesium ions in the AMP nanospheres additionally promoted the expression level of bone formation markers such as alkalin phosphatase (ALP), type I collagen (COL I), osteocalcein (OCN), and osteopontin (OPN) [21].

Although previous studies confirm the stimulating effect of Mg^{2+} on osteoblast proliferation, there is a lack of comparative studies analyzing the effect of simultaneous presence of magnesium and calcium ions on osteoblast growth and differentiation rate. As a result, there is a need for comparative studies like the present one. To the best of our knowledge, this study is the first one that specifically compares the effect of Mg^{2+} and Ca^{2+} ions on preosteoblast proliferation and differentiation. Here, the hypothesis tested was that Mg^{2+} and Ca^{2+} ions significantly increase proliferation rate of preosteoblasts and directs them towards osteoblastic differentiation *via* regulation of osteoblastic genes.

In this study, AMP particles were used as the Mg^{2+} source, and for the first time CMPs with Mg^{2+}/Ca^{2+} initial ratio of 1:1 were used as the source for Mg^{2+} and Ca^{2+} ions. Commercial HAs were used to provide preosteoblasts with Ca^{2+} ions. Cell compatibility and up-regulation of osteoblastic gene markers (ALP, Col I, OCN, OPN, RUNX2) were monitored and normalized to GAPDH housekeeping genes. Beta actin expression level was measured to confirm the accuracy of the results.

2. Experiments

2.1. Microwave assisted phosphate synthesis

All chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and used without further purification. The synthesis of AMP and CMP nanoparticles using the microwave assisted method has been reported in our previous publication [20]. Commonly used commercial hydroxyapatite was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and was used as a control. Stirring the reagents in the orders they are written (from left to right) (Table 1).

The solutions were then placed onto $10 \times 10 \times 1$ cm alumina insulating fiberboards and covered with an upside down 250 ml-capacity glass beaker. The entire assembly was later placed into a household microwave (MW) oven (Emerson, 800 W, 2450 MHz, Hackensack, NJ, USA) for 5 min. Once microwave heating was completed, the beakers were left to cool in the microwave for 15 min prior to transportation to the cold water bath for further cooling. The precipitates were magnetically stirred at 400 rpm in 500 ml of de-ionized water until the entire precipitates were dissolved. Finally, the solutions were washed with approximately 2 L of de-ionized water and filtered using a filter paper (Whatman Grade 4, 1004-055). The filtrates were then placed in an 80 °C oven overnight to dry. The synthesized AMP, CMP powders were then crushed and used for the analysis. The Mg/P molar ratio in AMP solution was set to 1 since the Mg/P molar ratio in human plasma/serum ranges from 3:2 to 2:3 [1,22]. The Mg^{2+}/Ca^{2+} ratio in CMP was 1:1.

3. Characterization

The morphological features of the as-synthesized particles were visualized by scanning electron microscope (SEM, S4800, Hitachi, Japan). Sample characterization was performed using X-ray diffraction (XRD) (Ulitma III, Rigaku, Woodlands, TX) with monochromated Cu Kα radiation, operated at voltage 40 kV and 44 mA setting over a 2θ range from 10 to 45 at a scanning speed of 1° per minute. Nanostructures of particles were investigated using transmission electron microscopy (TEM, HD-2300, Hitachi, Japan) with a voltage 200 kV.

Fourier transform infrared spectroscopy (FTIR, UMA-600 Microscope, Varian Excalibur Series, Holliston, MA, USA) was applied for chemical analysis of AMPs, CMPs, and commercial HAs. The transmittance of each sample was recorded with 256 scans with resolution of 4 cm^{-1} between 4000 and 700 cm^{-1}.

Table 2
Composition of 1 L test SBF.

Order	Reagent	SBF
1	NaCl	6.5456 g
2	$NaHCO_3$	2.2682 g
3	KCl	0.3727 g
4	Na_2HPO_4	0.1419 g
5	$MgCl_2 \cdot 6H_2O$	0.3045 g
6	1 M HCl	10 mL
7	$CaCl_2 \cdot 2H_2O$	0.3881 g
8	Na_2SO_4	0.072 g
9	Tris–Base	6.063 g
10	1 M HCl	33.3 mL