



Influence of ethanol content in the precipitation medium on the composition, structure and reactivity of magnesium–calcium phosphate



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ABSTRACT

Biocompatible amorphous magnesium calcium phosphate (AMCP) particles were synthesized using ethanol in precipitation medium from moderately supersaturated solution at pH 10. Some synthesis parameters such as, (Mg + Ca):P, Mg:Ca ratio and different drying methods on the structure and stability of as-produced powder was studied and characterized using SEM, XRD and cell cytocompatibility. The results showed that depending on the Mg²⁺ concentration, nano crystalline Struvite (MgNH₄PO₄·6H₂O) can also be alternatively formed. However, the as-formed AMCP preserved its amorphous structure after 7 days of incubation in SBF for tested phosphate concentration, and equally ionic concentration of magnesium and calcium.

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1. Introduction

This paper is motivated by our recent work on amorphous alkaline earth phosphates such as amorphous calcium phosphate (ACP) and amorphous magnesium phosphate (AMP) [1–3]. ACP is widely recognized as the intermediate phase in the synthesis of various crystalline calcium phosphate (CaP) phases. Starting off with initial research papers on the topic [4–6], various review papers have appeared on it including two recent ones [7,8]. These recent reviews clearly point out two important facts about the ACP materials: 1) they represent an important class of commercially important materials since their presence is important in products such as plasma sprayed coatings on implants to self-setting CaP cements; and 2) they have been well researched for a long time.

AMPs, on the other hand, have not received much attention. To the best of our knowledge, the contributions made by our group [2,3] are the first reports on the topic. The motivation for those two studies is derived from the wide recognition of Mg as well as magnesium phosphate (MgP) as important biomaterials with properties similar to or in many cases, better than CaP materials. While the surge in research of Mg based biodegradable metals is very significant, research on MgPs is also gaining momentum. For example, as opposed to CaP based cements, MgP cements have higher strength and biodegradability while maintaining all other desirable properties. Interestingly, none of these works considered AMPs. Previous studies on MgP synthesis did not vary the experimental parameters and examined the products [9].

Rather the focus was on fully crystalline MgP particles due to the difficulty in retaining MgP in unstable amorphous phase. In order to resolve this problem, we took advantage of a microwave assisted process [2]. However, the yield in such experiments is small.

This paper is a further extension of our work in order to prepare amorphous precipitates of alkaline earth phosphates of magnesium calcium phosphate using ethanol in precipitation medium. Being a simple process, precipitation has the potential to synthesize powders in large batches. Precipitation of amorphous compounds is facilitated at or above the neutral pH from moderately or highly supersaturated solution. For example, stable ACP can be prepared at pH = 10 [10,11]. It is generally accepted that ethanol in the precipitation solution helps in the formation of amorphous precipitates [10,12,13]. Some interesting features of the precipitation from ethanol containing solutions are given below. First, ethanol increases the degree of supersaturation of precipitating solution resulting in amorphous precipitates. Second, in the CaP system the presence of ethanol in precipitation solution decreases the level of protonated phosphate ions (HPO₄²⁻) and consequently decreases the Ca:P ratio [12]. Third, because of shielding of the water molecules, ethanol intensifies the interaction between ions remaining in the solution. Such interactions between PO₄³⁻, Mg²⁺, Ca²⁺ ions are a key element to forming amorphous phase at the early stage of precipitation, which is related to the ratio of these ions in the initial solution. Fourth, the relative concentrations of Mg²⁺ and Ca²⁺ in solution were seen to be a critical element during precipitation [14, 15]. Depending on the [Mg²⁺]:[Ca²⁺] in solution, a second stage of precipitation may begin by passing through an amorphous precursor. Thus, it is expected that the precipitates can be an amorphous matter or crystalline calcium phosphate or magnesium phosphate.

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The purpose of this study is to evaluate the precipitation of MCP under various experimental situations using ethanol. The effects of different ratios of (Mg + Ca):P, Mg:Ca and different drying methods on phases and morphologies of MCP precipitates were studied in order to find the optimum conditions in which the precipitation of amorphous compound of magnesium calcium phosphate (MCP) takes place.

2. Material and method

2.1. Synthesis in ethanol medium

The synthesis in ethanol medium was carried out under the conditions previously published by Rodrigues and Lebugle [12]. Various compositions with varying (Mg + Ca):P molar ratios and Mg:Ca ratios in solution were prepared as shown in Table 1. Solutions containing different concentrations of calcium nitrate tetrahydrate [$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$] and magnesium nitrate hexahydrate [$\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$] in 100 ml of deionized water and 100 ml of ethanol, were added rapidly, at 37 °C under stirring, to a solution containing diammonium hydrogen phosphate [$(\text{NH}_4)_2\text{HPO}_4$] in 250 ml of deionized water, 45 ml of ammonia (11 M) and 295 ml of ethanol (pH = 10.3). After formation, the pH values of the precipitate solution fell to 9.6–9.8. The precipitates were immediately centrifuged. The as-prepared materials were separated into 3 groups to study the impacts of different drying methods on material properties. One underwent 48 h freeze drying in order to promote the formation of amorphous phase. Another group was oven dried at 80 °C for 15 h to see whether they would end up with the amorphous product. The third was left at the ambient temperature for 72 h to prepare a room temperature sample to compare with other drying methods.

2.2. Characterization

The as-dried materials were studied by X-ray diffraction and scanning electron microscopy (SEM). The pattern was generally obtained with a (XRD) using mono chromated Cu radiation operated at a voltage of 40 kV and current of 44 mA, step width of 0.02 and scan speed of 1°/min setting. The morphological features of precipitates were visualized using a scanning electron microscope at 10 kV voltage (SEM, S4800, Hitachi, Japan) and all samples were gold coated for about 30 s before the SEM.

2.3. Stability testing

In order to study the stability of amorphous phase, powders were soaked in SBF solution. SBF was prepared following the recipe reported earlier [2]. The composition is shown in Table 2. The reagents were added and dissolved in solution one by one in the order listed in Table 2 and stored at 5 °C. Each 0.5 g dried powder was bottled in 40 ml of SBF separately. All samples were incubated in a 37 °C environment for 7 days. SBF was replenished every 48 h. Finally, powders were dried in an oven for further characterization.

Table 1
Chemical compositions of precipitation solution of various samples.

Sample code	Mg (mmol)	Ca (mmol)	P (mmol)	Mg:Ca	(Mg + Ca):P
H1	22.5	22.5	90	1	0.5
H3	22.5	22.5	30	1	1.5
H5	22.5	22.5	28	1	1.63
H6	22.5	22.5	22.5	1	2
H7	15	30	22.5	0.5	2
H8	30	15	22.5	2	2
H9	45	–	28	–	1.63
H10	–	45	28	–	1.63

Table 2
Compositions of 1 L test SBF.

Order	Reagents	SBF
1	NaCl	6.5456 g
2	NaHCO ₃	2.2682 g
3	KCl	0.3727 g
4	Na ₂ HPO ₄	0.1419 g
5	MgCl ₂ ·6H ₂ O	0.3045 g
6	1 M HCl	10 ml
7	CaCl ₂ ·2H ₂ O	0.3881 g
8	Na ₂ SO ₄	0.072 g
9	Tris-base	6.063 g
10	1 M HCl	33.3 ml

2.4. Cell viability assay and real-time RT PCR

MC3T3-E1 preosteoblast cells (ATCC, Manassas, VA, USA) were maintained in minimum essential medium alpha (MEM- α Thermo Scientific, Logan, UT, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 5% CO₂ and 37 °C. These cells were seeded in 6-well plates 24 h before the experiments. Serial composites were prepared as described previously, washed with ethanol, and immersed in the medium used to culture MC3T3-E1 cells. After 24 h, the conditioned medium was collected to replace the medium in the cell culture, and the cells were incubated at 5% CO₂ and 37 °C for another 24 h. To assess the cell viability, the cells were treated with thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) for 4 h, insoluble formazan dissolved in DMSO, and the optical density measured at 570 nm wavelength. To assess the expression of osteoblast genes, some cells were subject to RNA isolation after 72 h by the TRIzol method, and the RNA was reversely transcribed to prepared cDNA for real-time PCR using SsoFast EvaGreen supermix (Bio-Rad, Hercules, CA, USA). The primers targeted osteocalcin (OCN; forward 5'-GCAATAAGGTAGTGAACAGACTCC-3' and reverse 5'-CTTTGTAGGCGGTCTTCAAGC-3') and alkaline phosphatase (ALP; forward 5'-ATCTTTGGTCTGGC TCCATG-3' and reverse 5'-TTTCCCCTTCCCGTCCAC-3'). The sample harvested from cells grown in regular MEM- α served as a control.

3. Results

3.1. Effect of different drying methods on phase formation

To evaluate the effect of drying method on precipitates, MCP precipitates of H5 composition were dried via freeze drying, oven drying and under ambient temperature condition. As shown in the XRD diffraction pattern in Fig. 1, sample H5 dried via freeze dryer and ambient temperature consisted of crystalline Struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$). It has been reported that precipitation of Struvite is feasible at the ratio of $[\text{Mg}^{2+}]:[\text{NH}_4^+]:[\text{PO}_4^{3-}]:1:1:1$ and pH = 9 [15,16]. It should be noted that the relative intensity of the peaks for samples dried at ambient temperature correspond to Struvite is relatively higher than the freeze drying samples. This suggests changes in the texture of the crystalline material and more specifically changes in the crystallite size [15], and reflecting crystal growth during drying at ambient temperature. To study the crystal growth, the crystallite size was measured using Jade™ software (Materials Data Incorporated). The crystallite size of freeze-dried sample is 135.6 nm compared to >500 nm for samples dried under ambient condition. The peak at $2\theta = 29.41^\circ$ appeared for oven dried sample corresponds to (2 1 0) reflection of Struvite that shows a crystallite size of 78.4 nm.

3.2. Effect of (Mg + Ca):P ratio on phase formation

Fig. 2 represents the effect of the varying ratio of (Mg + Ca):P with regard to amorphous matter precipitation. The constant ratio of

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