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Biocompatibility of magnesium phosphate minerals and their stability under physiological conditions

Faleh Tamimi [a,*], Damien Le Nihouannen [a], David C. Bassett [a], Suzette Ibasco [a], Uwe Gbureck [b], Jonathan Knowles [c,e], Adrian Wright [d], Andrew Flynn [a], Svetlana V. Komarova [a], Jake E. Barralet [a]

[a] Faculty of Dentistry, McGill University, Montreal, Canada
[b] Department for Functional Materials in Medicine and Dentistry, University of Würzburg, Würzburg, Germany
[c] Eastman Dental Institute, University College London, London, UK
[d] School of Chemistry, University of Birmingham, Birmingham, UK
[e] WCU Research Centre of Nanobiomedical Science, Dankook University, San#29, Anseo-dong, Dongnam-gu, Cheonan-si, Chungnam, 330-714 South Korea

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ABSTRACT

Magnesium phosphates such as newberyite ($MgHPO_4{\cdot}3H_2O$) are formed in vivo and are known to be biodegradable and nontoxic after implantation. Indeed, magnesium apatites have been shown to support osteoblast differentiation and function, and bone formation can occur around metallic magnesium implants. However, very little is known regarding the precipitation and stability of magnesium phosphates in physiological environments. In order to address this, the aqueous formation of magnesium phosphate as a function of pH, temperature and ion concentration is reported. Physicochemical characterization of the precipitates was carried out; additionally, biocompatibility and gene expression of osteoblast differentiation markers for bone formation via an in vitro cell culture assay were determined. Precipitation conditions for newberyite, tribasic magnesium phosphate pentahydrate, holtedahlite, bobierrite and cattiite were determined. Under physiological conditions of pH, temperature and magnesium phosphate concentration, no precipitates were formed. However, at concentrations 10–100 times higher than physiological, magnesium phosphate precipitates of cattiite and newberyite were formed. These two minerals demonstrated biocompatibility with osteoblast cultures and induced osteoblast adhesion and differentiation. The pattern of expression of OCN and CollA1 genes in the presence of newberyite crystals was comparable to that of calcium phosphate bioceramics. In our experiments, we have shown that certain magnesium phosphate phases such as newberyite and cattiite are able to promote in vivo osteogenic activity in a similar way to calcium phosphates such as hydroxyapatite and brushite. This confirms the great potential of magnesium phosphate ceramics in the development of new biomaterials for bone regeneration.

1. Introduction

For over 40 years bioceramic research has been dominated by studies of calcium phosphates in attempts to develop reliable synthetic bone graft substitutes [1]. Several calcium phosphate-based biomaterials, based on either tricalcium phosphate or hydroxyapatite, have been approved by the US Food and Drug Administration for clinical use; however, no material has been found to date that is ideal in terms of osteoconduction and tissue bonding [2,3]. Magnesium phosphates may occur in physiological and pathological mineralized tissues [4,5]. Whitlockite (β-tricalcium magnesium phosphate) can be found in salivary gland stones, as well as in dental calculi [4], while struvite ($MgNH_4PO_4{\cdot}6H_2O$) and newberyite ($MgHPO_4{\cdot}3H_2O$) are found in kidney stones [5]. Interestingly, these phases can be synthesized into materials showing promising properties for hard tissue regeneration [6]. One of the first degradable orthopaedic implants ever studied was made of magnesium metal [7]. However, magnesium is reactive in aqueous conditions and can produce hydrogen gas. Therefore development of magnesium-based biomaterials faltered until development of new alloys for biodegradable cardiovascular stents [8]. Recently the development of magnesium phosphate cements for orthopaedic applications has been reported [6]. To date there is very little information regarding the precipitation conditions, stability and cytotoxicity of magnesium phosphates, which are essential in determining the biological properties of the various phases.

Generally magnesium phosphates are precipitated from stoichiometric mixtures of Mg^{2+} and PO_4^{3-} ions at ratios of 3:2 and 1:1 in order to obtain neutral and acid magnesium phosphates [9]. However, the ratio of magnesium to phosphate ions in vivo is very different; for

* Corresponding author. Tel.: +1 5143987203.
E-mail address: faleh.tamimimarino@mcgill.ca (F. Tamimi).

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instance, Mg^{2+} concentration in serum is 0.7–0.9 mM while PO_4^{3-} concentration is 0.9–1.4 mM, giving a ratio of Mg^{2+}:PO_4^{3-} of ~2:3 [10,11]. Very little is known about the stability of magnesium phosphate compounds in physiological conditions and, despite low ionic concentrations, certain magnesium phases may still form [12,13].

To our knowledge, no previous study has been undertaken to explore the precipitation conditions of magnesium phosphates under physiological conditions and their compatibility with bone cells. This information is essential in order to optimize strategies for the production of new magnesium phosphate-based biomaterials, as well as for a better understanding of their stability in vivo.

In this study, phases of precipitated magnesium phosphates were mapped over a range of temperatures, pHs, concentrations and Mg^{2+}:PO_4^{3-} ratios. The resulting precipitates were characterized in terms of crystallinity, morphology and solubility; biocompatibility was determined with osteoblasts and bone marrow cell cultures.

2. Materials and methods

2.1. Precipitation

All reagent grade chemicals were purchased from Sigma–Aldrich Inc. (St Louis, MO) and used without further purification.

The stability of magnesium phosphate solutions was tested by mixing magnesium chloride and hydrogen phosphate solutions in a physiological molar ratio of Mg^{2+}:PO_4^{3-} of 2:3 [10]. The solutions were prepared with three different concentrations of magnesium and phosphate: (i) physiological concentrations (0.67 mM Mg^{2+}: 1.0 mM PO_4^{3-}); (ii) solutions 10× more concentrated than physiological (6.7 mM Mg^{2+}:10 mM PO_4^{3-}); and (iii) solutions 100× more concentrated than physiological (67 mM Mg^{2+}:100 mM PO_4^{3-}). The temperature of the solutions was maintained at 4, 21, 37, 55 and 75 °C using a refrigerator (4 °C), room temperature (21 °C), water bath (37 and 55 °C) and an oven (75 °C), while the pH was adjusted (5.0–12.0) prior to mixing with 1 M HCl and NaOH. All solutions were buffered with tris-(hydroxymethyl) aminomethane (0.1 g per 1000 ml). After initial mixing, the solutions were thoroughly stirred, and left to age for 24 h at fixed temperature. The resulting precipitates were then centrifuged, washed in deionized water and dried overnight in a vacuum oven at 37 °C.

The effect of the Mg^{2+}:PO_4^{3-} ratio was studied under physiological conditions (37 °C; pH 7.4) while maintaining a PO_4^{3-} concentration of 100 mM. Solutions with a Mg^{2+}:PO_4^{3-} of 1:1, 1:2 and 1:3 were prepared, and the resulting precipitates were collected for further characterization.

2.2. Characterization

X-ray diffraction (XRD) analysis of the precipitates was performed to evaluate their phase composition. A vertical-goniometer X-ray diffractometer (Philips model PW1710, Bedrijven b. v. S&I, The Netherlands) equipped with a Cu radiation source was used for the powder diffraction pattern collection. Data were collected from 2θ of 10°–80°, with a step size of 0.02° and a normalized count time of 1 s per step. The phase composition was compared with the International Centre for Diffraction Data reference patterns.

The Fourier transform infrared spectroscopy (FTIR) absorbance spectra of the precipitates were recorded with a DTGS-KBr detector and KBr beam splitter with 32 scans at a resolution of 0.1 cm^{-1} (Nexus 470 Thermo-Nicolet, Thermo Fisher Scientific, Waltham, MA). The spectrum of powdered sample in KBr medium was recorded in the range from 350 to 3000 cm^{-1}.

Samples were coated in gold–palladium alloy before observation using a scanning electron microscope (JEOL JSM-840A at 15 kV, JEOL, Japan) and energy-dispersive X-ray spectroscopy (EDX) was carried out using an EDAX detector (EDAX, Mahwah, NJ).

Precipitate density was measured using a helium pycnometer (Accupyc 1330; Micromeretics; Bedfordshire, UK) and Mg^{2+}:PO_4^{3-} ratio was measured with inductively coupled plasma mass spectroscopy (ICP-MS). Samples ($n = 3$ per condition) were analysed against Varian ICP-MS standard solutions (Merck, Darmstadt, Germany). Solubility of the magnesium phosphate species was measured by detecting the Mg^{2+} ion concentration at equilibrium in aqueous solution using ion chromatography (Dionex co., Sunnyvale, CA). Simultaneous thermogravimometric analysis (TGA) and differential scanning calorimetry (DSC) was performed with a combined TGA/DSC instrument (SDT Q600, TA Instruments, New Castle, DE). Samples were heated from 10 to 1000 °C at a rate of 10 °C min^{-1}. During heating, the temperature was maintained at 100 °C of 60 min.

2.3. Cell culture

2.3.1. Osteoblast cell culture

The murine pre-osteoblastic cell line MC3T3-E1 is a non-transformed cell line established from newborn mouse calvaria and exhibiting an osteoblastic phenotype, and was obtained from the American Type Culture Collection (Rockville, MD, USA) [21]. MC3T3-E1 cells were routinely cultured in 25 cm^2 tissue culture flasks in a-minimal essential medium with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin and 1% sodium pyruvate at 37 °C in a humidified atmosphere containing 5% CO_2 in air. Cells were subcultured once a week using trypsin/EDTA and maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Fresh medium was added every 2 days.

2.3.2. Mouse bone marrow cells

All animal studies were performed in accordance with the McGill University guidelines established by the Canadian Council on Animal Care. Mice (C57BL6/J, male, 6 weeks old) were purchased from Charles River (Charles River Co., USA). Mouse-derived bone marrow cells were collected from mouse tibia and femora as described previously [14]. Briefly, femora and tibia were dissected under sterile conditions and the surrounding muscles were detached from the bones. Tibia and femurs were cut in half and the bone marrow was flushed with minimum essential medium (MEM), containing 10% serum and 1% antibiotics, dispensed by a 10 ml syringe with a 26-gauge needle. Bone marrow was flushed from the tibia and femora under aseptic conditions. Mouse-derived bone marrow cells (mBMCs) were then cultured in 75 cm^2 tissue culture flasks (2.5×10^6 cells cm^{-2}) in MEM (Wisent Inc., Montreal, Canada; Cat. No. 310-022-CL) with 10% serum (HyClone; Cat. No. SH 30396-03, Fisher Scientific, Ottawa, Canada), 1% penicillin/streptomycin antibiotics (Cat. No 450-201-EL; Wisent Inc., Canada), 1% sodium pyruvate (Cat. No. 600-110-EL; Wisent) and 50 μg ml^{-1} L-ascorbic acid (Sigma–Aldrich Co., St Louis, MO, USA; Cat. No. A5960). After 7–10 days, cells were detached with trypsin/EDTA (Cat. No. 325-042-EL; Wisent) and plated at a density of 10^4 cells cm^{-2} onto the surface/layer of materials or directly onto the tissue culture-treated polystyrene (Corning Life Sciences, Lowell, MA, USA).

2.4. Cytotoxicity test

Cytotoxicity of the magnesium phosphate powders was evaluated by measuring the lactate dehydrogenase (LDH) produced by MC3T3-E1 cells using a cytotoxicity kit (Cytotoxicity Detection Kit^{Plus}, Roche Applied Science, USA). Briefly, MC3T3-E1 cells were seeded into 96-well plates with 10 mg of the magnesium phosphate precipitates at a final density of 10^4 cells $well^{-1}$ and incubated for 12 h at 37 °C in an atmosphere of 5% CO_2. MC3T3-E1

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