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Elucidating the individual effects of calcium and phosphate ions on hMSCs by using composite materials

Charlène B.S.S. Danoux [a], David C. Bassett [b,1], Ziryan Othman [a], Ana I. Rodrigues [a,c,d], Rui L. Reis [c,d], Jake E. Barralet [b], Clemens A. van Blitterswijk [a,2], Pamela Habibovic [a,*,2]

[a] Department of Tissue Regeneration, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands
[b] Faculty of Dentistry, McGill University, 3640 University Street, Montreal, QC H3 A 2B2, Canada
[c] 3B's Research Group, Universidade do Minho, AvePark, 4806-909 Caldas das Taipas, Portugal
[d] ICVS/3B's – PT Government Associated Laboratory, Guimarães, Portugal

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ABSTRACT

The biological performance of bone graft substitutes based on calcium phosphate bioceramics is dependent on a number of properties including chemical composition, porosity and surface micro- and nano-scale structure. However, in contemporary bioceramics these properties are interlinked, therefore making it difficult to investigate the individual effects of each property on cell behavior. In this study we have attempted to investigate the effects of calcium and inorganic phosphate ions independent from one another by preparing composite materials with polylactic acid (PLA) as a polymeric matrix and calcium carbonate or sodium phosphate salts as fillers. Clinically relevant bone marrow derived human mesenchymal stromal cells (hMSCs) were cultured on these composites and proliferation, osteogenic differentiation and ECM mineralization were investigated with time and were compared to plain PLA control particles. In parallel, cells were also cultured on conventional cell culture plates in media supplemented with calcium or inorganic phosphate to study the effect of these ions independent of the 3D environment created by the particles. Calcium was shown to increase proliferation of cells, whereas both calcium and phosphate positively affected alkaline phosphatase enzyme production. QPCR analysis revealed positive effects of calcium and of inorganic phosphate on the expression of osteogenic markers, in particular bone morphogenetic protein-2 and osteopontin. Higher levels of mineralization were also observed upon exposure to either ion. Effects were similar for cells cultured on composite materials and those cultured in supplemented media, although ion concentrations in the composite cultures were lower. The approach presented here may be a valuable tool for studying the individual effects of a variety of soluble compounds, including bioinorganics, without interference from other material properties.

1. Introduction

Calcium phosphate-based compounds have a long history of use in bone replacement and regeneration, mainly owing to their chemical likeness to bone mineral [1]. As discussed in a recent review, inorganic bone graft substitutes are generally considered to play a beneficial role in bone repair only in the solid state [2]. In other words, their main function is "structural", meaning that they act as a barrier to soft tissue infiltration, offer temporary or lasting mechanical support to damaged hard tissues and facilitate the onset and growth of new bone on their surface.

It is also important to consider that all calcium phosphate-based compounds are, to varying extents, degradable in the physiological environment, either by physico-chemical dissolution or through cellular activity [3]. During the process of degradation, structural function of the substitute is diminished, but this process is accompanied by release of calcium and phosphate ions. Indeed, it is believed that in this process of calcium and phosphate release, followed by the re-precipitation of a carbonated apatite layer, along with co-precipitation of endogenous proteins, lies the origin of bioactivity of calcium phosphates [4]. A majority of the literature regarding calcium phosphate bioactivity focuses on the formation of this biological apatite layer, and the *in vivo* effects of free calcium (Ca^{2+}) and phosphate (Pi) ions both indi-

* Corresponding author.
E-mail address: p.habibovic@maastrichtuniversity.nl (P. Habibovic).
[1] Current affiliation: Department of Physics, Norwegian University of Science and Technology, Trondheim 7491, Trondheim, Norway.
[2] Current affiliation: MERLN-Institute for Technology-Inspired Regenerative Medicine, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands.

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vidually and in combination are largely ignored [5]. Ca^{2+} and Pi are considered to be inorganic ions with therapeutic potential, also called bioinorganics. The interest for bioinorganics as a synthetic and potentially safer alternative to biological growth factors is increasing as a method to improve the biological performance of bone graft substitutes, while retaining their synthetic character [2].

Several examples of *in vitro* studies investigating the effects of Ca^{2+} and Pi on growth and differentiation of cells relevant for bone repair and regeneration can be found. For example, increase of calcium ion concentration in cell culture medium has been shown to enhance the proliferation and osteogenic differentiation of both osteoblasts [6] and human periosteum derived stem cells (hPDCs) [7] in a dose-dependent manner. Elevated Ca^{2+} concentration [7.8 mM] in hMSC culture resulted in upregulation of a number of osteogenic markers, including bone morphogenetic protein-2 (BMP-2), osteocalcin (OC), osteopontin (OP) and bone sialoprotein (BSP) [8]. It has recently been shown that stimulation of osteogenic differentiation of osteoprogenitor cells by calcium occurs by signaling through L-type calcium channels rather than through calcium sensing receptors [8,9].

The inorganic phosphate ion concentration in medium has also been shown to influence osteoprogenitor cell fate in a dose dependent manner. For example, a 96-h exposure to 7 mM Pi, led to apoptosis of primary human osteoblast-like cells, induced by a mitochondrial membrane permeability transition caused by the anion [10]. However, a Pi concentration between 2 and 10 mM was also shown to support hPDC proliferation and osteogenic differentiation in a dose-dependent manner [7] and acts as a specific signal for the induction of OP gene expression in osteoblasts [11]. In a recent study by Shih et al. it was demonstrated that extracellular phosphate uptake through SLC20a1, a sodium phosphate symporter, supports osteogenic differentiation of hMSCs via adenosine, an ATP metabolite, which acts as an autocrine/paracrine signaling molecule through the A2b adenosine receptor [12].

The use of conventional calcium phosphate ceramic bone graft substitutes does not allow for in depth studies of the effects of individual physicochemical parameters on the bioactivity of the material. During degradation, release of calcium ions is naturally also accompanied by a release of phosphate ions. Furthermore, surface structural properties, such as macro- and micro-porosity, specific surface area, roughness [13–16] and overall geometry [17–19], may affect the biological performance in terms of osteoconductivity and osteoinductivity, either independently or partially dependently of the degradation properties. In order to isolate chemical effects from physical and structural effects, one of our earlier studies involved loading highly soluble inorganic phosphate salts into an inert polymeric delivery vehicle that was implanted intramuscularly in mice. This study showed that local supersaturation of soft tissue surrounding the implant with inorganic phosphate resulted in extensive collagen mineralization proximal to the implant [20].

To further explore the potential of polymeric carriers as delivery vehicles for bioinorganics, and to study the effects of calcium or phosphate released from such carriers independently, composite materials, consisting of PLA, a biocompatible and biodegradable aliphatic polyester, and calcium or phosphate salts were developed in this study. These composites were shaped into particles, offering a three-dimensional substrate for the culture of hMSCs and simultaneously acting as calcium- or phosphate-delivery systems over 14 days of culture. In parallel, hMSCs were cultured in media supplemented by Ca^{2+} or Pi. We assessed the independent effects of Ca^{2+} and Pi on hMSC proliferation, differentiation toward osteogenic lineage and extracellular matrix (ECM) mineralization.

2. Materials and methods

2.1. Synthesis of composite materials

Calcium carbonate was selected as a source of calcium ions (Fisher Biotech, USA). A sedimentation cut-off was performed in ethanol in order to select salt particles with a maximum diameter of 35 μm. A combination of sodium phosphate monobasic (25 wt.%) and dibasic (75 wt.%) salts (Fisher Biotech, USA) was prepared to obtain a pH neutral source of inorganic phosphate ions. This mixture was ground in a pestle and mortar and sieved through a 38 μm sieve. The polymeric phase of the composites used in this study consisted of amorphous poly(D,L-lactic acid) (PLA) (Purac, Gorinchem, the Netherlands) with a molecular weight of 59,000 Da.

The three composites, i.e. composite containing 5 wt.% calcium carbonate salt (PLA-Ca5%), composite containing 50 wt.% calcium carbonate salt (PLA-Ca50%) and composite containing 5 wt.% sodium phosphate salt mixture (PLA-Pi5%) (Table 1), and PLA controls were prepared as follows: salts were dispersed and PLA was dissolved in chloroform. The mixture was then added drop by drop using a separating funnel to isopropanol under constant stirring. The precipitate was collected and rinsed overnight in isopropanol, drained and dried. The material was placed in a silanized glass dish and dried further at 150 °C and at a pressure of 10 in Hg for 5 min in order to remove solvent traces. Finally, the material was ground and sieved to collect particles between 0.5 and 1 mm in diameter. It should be noted that we were able to develop a composite with a higher (20 wt.%) sodium phosphate salt content; however, a burst release of Pi was observed. This burst release was overcome by the addition of PLA with a higher molecular weight, but since this change in polymeric carrier would not allow a proper comparison with other conditions, we decided not to include it in the study.

2.2. Material characterization

Salt grains and composite particles were sputter coated with Au/Pd and observed by scanning electron microscopy (FE-SEM, Hitachi S-4700; ESEM-FEG, Philips XL30) under secondary electron mode with an acceleration voltage of 10 kV. Salt incorporation and homogeneity of distribution in the polymeric phase were assessed using SEM and Energy Dispersive X-ray Spectroscopy (EDAX; Apollo X, Ametek).

2.3. Ion release and particle degradation

Ion release from the composite particles was assessed over 2 weeks in a buffer of trishydroxymethyl aminomethane (0.1 M TRIS ($(CH_2OH)_3CNH_2$), 0.15 M NaCl, adjusted to a pH of 7.3 with 1 M HCl). 100 mg of particles were immersed in 15 mL of TRIS buffer and maintained at 37 °C in a still water bath. Upon immersion in the solution, particles of all material types settled at the bottom of the vial. The calcium and phosphate content of the buffer was analyzed by ion chromatography (DX2500, Dionex, Sunnyvale,

Table 1
Composition of the composites and controls.

Materials	PLA 59,000 Da (wt.%)	Calcium salt (wt.%)	Phosphate salt (wt.%)
PLA-Ca5%	95	5	
PLA-Ca50%	50	50	
PLA-Pi5%	95		5
PLA	100		

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