



## The in vivo performance of CaP/PLGA composites with varied PLGA microsphere sizes and inorganic compositions



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### ABSTRACT

Enrichment of calcium phosphate (CaP) bone substitutes with poly(lactic-co-glycolic acid) (PLGA) microspheres to create porosity overcomes the problem of poor CaP degradation. The degradation of CaP–PLGA composites can be customized by changing the physical and chemical properties of PLGA and/or CaP. However, the effect of the size of dense (solid rather than hollow) PLGA microspheres in CaP has not previously been described. The present study aimed at determining the effect of different dense (i.e. solid) PLGA microsphere sizes (small (S)  $\sim 20\ \mu\text{m}$  vs. large (L)  $\sim 130\ \mu\text{m}$ ) and of CaP composition (CaP with either anhydrous dicalcium phosphate (DCP) or calcium sulphate dihydrate (CSD)) on CaP scaffold biodegradability and subsequent bone in-growth. To this end mandibular defects in minipigs were filled with pre-set CaP–PLGA implants, with autologous bone being used as a control. After 4 weeks the autologous bone group outperformed all CaP–PLGA groups in terms of the amount of bone present at the defect site. On the other hand, at 12 weeks substantial bone formation was observed for all CaP–PLGA groups (ranging from  $47 \pm 25\%$  to  $62 \pm 15\%$ ), showing equal amounts of bone compared with the autologous bone group ( $82 \pm 9\%$ ), except for CaP with DCP and large PLGA microspheres ( $47 \pm 25\%$ ). It was concluded that in the current study design the difference in PLGA microsphere size and CaP composition led to similar results with respect to scaffold degradation and subsequent bone in-growth. Further, after 12 weeks all CaP–PLGA composites proved to be effective for bone substitution.

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### 1. Introduction

In the reconstructive fields of orthopaedics and oral-maxillofacial surgery, bone substitutes based on calcium phosphates (CaP) are widely used [1–5]. When applied in a clinical setting, mouldable or injectable CaP-based materials have several advantages over prefabricated solid CaPs, including ease of handling, application through minimally invasive surgical techniques, and the ability to be fully adapted to the dimensions of the bone defect [6]. The application of injectable CaP cements (CPCs) was already reported in 1950 [7], although the first medical application was reported only in 1972 [8], and patented in 1985 by Brown and Chow [9]. CPCs are prepared by mixing a powder and a liquid component, after which in situ hardening occurs via a near isothermic reaction upon application in vivo [10].

The main advantage of CaP-based materials is the excellent biocompatibility due to their chemical similarity to the natural mineral of bone tissue [4,10]. Hydroxyapatite (HA) represents the most used CaP-based phase for biomedical applications, despite its slow degradation rate that allows only limited replacement by newly formed bone tissue [4,10,11]. Incorporation of poly(lactic-co-glycolic acid) (PLGA) microspheres to create porosity can overcome the problem of poor CaP degradation [10]. After placement in vivo these PLGA microspheres degrade relatively rapidly, resulting in a porous CaP scaffold [12]. The increase in porosity after PLGA microsphere degradation substantially enlarges the CaP surface area in contact with cells and, hence, accelerates the degradation of CaP-based materials, allowing their gradual replacement by bone tissue [13]. By changing the physical and chemical properties of PLGA and CaP, tailor-made CaP–PLGA degradation becomes feasible. For PLGA these properties are (from least to most influential) molecular weight, end-group functionalization, monomer ratio and microsphere structure, as well as morphology [6,13–19].

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With respect to microsphere structure, dense PLGA microspheres have been shown by Félix-Lanao et al. [13,19] to evoke enhanced CaP–PLGA degradation compared with hollow PLGA microspheres in a rabbit femoral condyle model. This was explained by a greater amount of acidic degradation products from dense PLGA microspheres compared with hollow PLGA microspheres [19], resulting in faster dissolution of CaP. Further, pore size proved to have an influence on CaP degradation and subsequent bone in-growth [20,21], which can be easily controlled by varying the PLGA microsphere size [22]. Considering microsphere morphology, a minimal CaP implant pore size of 50–100  $\mu\text{m}$  with interconnections of 20  $\mu\text{m}$  is suggested in the literature for bone in-growth [20,21,23,24], since relatively larger pores favour direct osteogenesis by allowing cell distribution, vascularization and high oxygenation [21]. Even so, Karageorgiou and Kaplan observed that greater pore sizes result in a higher CaP surface area interacting with the host tissue, which can accelerate CaP degradation [21]. In contrast, it should be noted that there are indications that CaP–PLGA composites containing hollow PLGA microspheres with dimensions of  $\sim 20 \mu\text{m}$  are able to support the bridging of a bone defect [5,13]. This is explained by the fact that small hollow PLGA microspheres will result in an increase in acidic degradation products per unit volume compared with large PLGA microspheres (i.e. the volumetric proportion of PLGA in hollow PLGA microspheres is higher for small microspheres compared with large microspheres). When combining the effects of PLGA microsphere structure and morphology, it can be assumed that the described effect of PLGA microsphere structure (i.e. dense vs. hollow) can be influenced by differences in microsphere size. Such an effect of the size of dense PLGA microspheres on CaP degradation and subsequent bone in-growth has not been studied previously to the best of our knowledge.

With respect to CaP composition,  $\alpha$ -tricalcium phosphate ( $\alpha$ -TCP)-based CPC–PLGA containing anhydrous dicalcium phosphate (DCP) can be completely degraded in vivo [5,13,14]. Alternatively,  $\alpha$ -TCP-based CPC containing calcium sulphate dihydrate (CSD) is markedly more soluble than  $\alpha$ -TCP-based CPC containing DCP. Since body fluids are more readily saturated towards DCP [25], CSD is expected to dissolve more rapidly after implantation [26,27] and, hence, is expected to release more calcium ions and promote more bone formation.

The present study aimed at evaluating the effect of different dense PLGA microsphere sizes on (i) biodegradability of and (ii) subsequent bone in-growth into CPC containing either DCP or CSD. To this end an in vivo experiment was performed in a minipig mandibular defect model, in which CPC–PLGA pre-set discs were implanted. Both small ( $\sim 20 \mu\text{m}$ ) and large ( $\sim 130 \mu\text{m}$ ) dense PLGA microspheres were used with CPCs with DCP or CSD, while autologous bone discs were used as a control. The hypothesis was that large dense PLGA microspheres would outperform small dense PLGA microspheres (due to greater amounts of acidic degradation products per volume unit) and CSD would outperform DCP (due to the stability of the CaP phase) with respect to CaP degradation and subsequent new bone formation.

## 2. Materials and methods

### 2.1. Materials

CPC containing DCP consisted of 85%  $\alpha$ -TCP (CAM Bioceramics BV, Leiden, The Netherlands), 10% DCP (Sigma–Aldrich, St Louis, MO) and 5% precipitated hydroxyapatite (PHA) (Merck, Darmstadt, Germany). A 0.2  $\mu\text{m}$  filter sterilized aqueous solution of 2%  $\text{Na}_2\text{HPO}_4$  (Merck) was used as the liquid phase for CPC (liquid/powder ratio 0.39  $\text{ml g}^{-1}$ ).

The CPC containing CSD was based on a previously described formulation [26] and consisted of 80%  $\alpha$ -TCP (RMS Foundation, Bettlach, Switzerland), 9% CSD (Fluka, Buchs, Switzerland), 10% PHA and 1% disodium hydrogen phosphate (DSHP) (Fluka). A 0.5% sodium hyaluronate solution was used as the liquid phase (liquid/powder ratio 0.46  $\text{ml g}^{-1}$ ) (Vitrolife AB, Gothenburg, Sweden). Both the CPC powder and the sodium hyaluronate solution were kindly provided by the RMS Foundation (Bettlach, Switzerland).

PLGA (Purasorb® PDLG 5002A, molecular weight  $\sim 17 \text{ kDa}$ , acid terminated end-group functionalization with a lactic acid/glycolic acid ratio of 50:50) was obtained from Purac Biomaterials BV (Gorinchem, The Netherlands).

### 2.2. Preparation of PLGA microspheres

Dense PLGA microspheres were prepared by a single emulsion technique as previously described [13]. Briefly, 0.4 g of PLGA was dissolved in 4 ml of dichloromethane (DCM) (Merck) for small microspheres, while 0.67 g of PLGA was dissolved in 3 ml of DCM for large microspheres. Both solutions were transferred to beakers each containing 150 ml of 0.3% polyvinylalcohol solution (Acros Organics, Geel, Belgium) with stirring. After 5 min of stirring, 100 ml of a 2% isopropanol solution (Merck) was added to each beaker and stirred for 1 h. The microspheres were allowed to settle for 1.5 h and the supernatant was decanted. The microspheres were washed twice with demineralized water, frozen at  $-20 \text{ }^\circ\text{C}$ , freeze-dried for 24 h and finally stored at  $-20 \text{ }^\circ\text{C}$ .

### 2.3. Preparation of CPC–PLGA pre-set discs

Both types of CPC powders were mixed with either small (S) or large (L) PLGA microspheres in a 69/31 wt.% ratio to a total weight of 1.16 g in a 2 ml syringe with a closed tip (BD Plastipak™, Becton Dickinson SA, Madrid, Spain), based on the results of previous experiments [1]. In this way the materials DCP-S, DCP-L, CSD-S and CSD-L were created.

After adding the liquid components to the syringes, the CPC–PLGA composites were mixed for 30 s with a standard amalgam mixer (Silamat®, Vivadent, Schaan, Liechtenstein) and immediately used to fill a polytetrafluorethylene (PTFE) mould with specified cylindrical holes (diameter 7.0 mm, height 4.0 mm). The centre of each scaffold was marked with a titanium pin (diameter 0.5 mm, length 4 mm) for X-ray guided sample retrieval, after which the CPC–PLGA scaffolds were left to set at room temperature for 24 h and subsequently sterilized by  $\gamma$ -irradiation with a minimum dose of 25 kGy (Isotron BV, Ede, The Netherlands).

### 2.4. Microsphere and CPC–PLGA characterization

The microsphere size distribution was assessed by light microscopy (Leica/Leitz DM RBE Microscope system, Leica Microsystems AG, Wetzlar, Germany) and digital image software (Leica QWin Pro®, Leica Microsystems AG) using a sample size of  $>200$  PLGA microspheres. The CPC–PLGA discs were produced with a macroporosity of  $\sim 55\%$ , following a previously described protocol [1].

### 2.5. In vivo experiment

All in vivo work was conducted in the Central Animal Laboratory, Beijing Stomatological Hospital, China Capital Medical University, approved by the local ethical standards and guidelines for the care and use of laboratory animals. The experimental groups included the four pre-set disc formulations (i.e. DCP-S, DCP-L, CSD-S and CSD-L) as well as autologous bone (AUT) as a positive control.

ID	Title	Pages
547	The in vivo performance of CaP/PLGA composites with varied PLGA microsphere sizes and inorganic compositions	9

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