Application of poly(trimethylene carbonate) and calcium phosphate composite biomaterials in oral and maxillofacial surgery
Zeng, Ni

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chapter

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EVALUATION OF OSTEOINDUCTIVITY OF DIFFERENT CALCIUM PHOSPHATE AND PTMC-CALCIUM PHOSPHATE COMPOSITE BIOMATERIALS IN A SHEEP MODEL

To be submitted
ABSTRACT

Osteoinduction refers to *de novo* bone formation induced by biomaterials in places where physiologically no bone tissue is formed. Biomaterials with osteoinductive capacities have been shown to fill bone defects of critical sizes with ubiquitous new bone formation. Therefore, osteoinduction has been regarded as an important characteristic for biomaterials aiming at bone regeneration and is determined by various factors. In our study, we tested osteoinductive capacities of different calcium phosphate bioceramic particles and porous poly(trimethylene carbonate)(PTMC)-calcium phosphate composite scaffolds in a sheep model. Biphasic calcium phosphate (BCP) particles of 45-150 µm and 150-500 µm, microporous β-tricalcium phosphate (β-TCP) particles of 45-150 µm, non-microporous β-TCP particles of 45-150 µm and 150-500 µm were implanted in sheep long dorsal muscle for three and nine months. Likewise, porous composite scaffolds, in which BCP particles, microporous β-TCP particles and β-TCP particles, all of 45-150 µm, had been incorporated into PTMC matrices, were implanted in sheep long dorsal muscle for three and nine months. Porous PTMC scaffolds were implanted as controls. Abundant new bone formation was induced by BCP particles of both size ranges, while no new bone formation was induced by the other biomaterials. Implantation of the abovementioned biomaterials led to uneventful degradation of the PTMC matrices and the incorporated calcium phosphate particles, and provoked no obvious tissue reaction. Further studies are needed to produce composite biomaterials with osteoinductive capacities.
INTRODUCTION

New bone formation in physiological remodeling and repairing of damaged bone tissue occurs via osteogenesis, which involves recruiting mesenchymal stem cells from blood and inducing their proliferation and differentiation to osteoblasts at the sites(1). Concerning reconstructions of bone defects by biomaterials, the term ‘osteoconduction’ is used to describe new bone formation stimulated and guided on surfaces or into pores provided by the biomaterials implanted in bone defects. Besides, a phenomenon called ‘osteoinduction’, defined as “the induction of undifferentiated inducible osteoprogenitor cells that are not yet committed to the osteogenic lineage to form osteoprogenitor cells”(2), describes induced new bone formation in the biomaterials implanted in ectopic sites, such as subcutaneous or intramuscular implantations(3). Biomaterials with osteoconductive properties ‘promote the recruitment and migration of osteogenic cells into the wound site’(4) and serve as scaffolds for new bone formation to occur, while biomaterials with osteoinductive properties actively induce new bone formation and are believed to have the essential ability to heal bone defects of critical sizes successfully(5). Compared to what occurs in osteoconductive biomaterials, new bone formation induced by osteoinductive biomaterials occurs not only at the interface between the biomaterials and the host tissue, but also all over the defects filled with the biomaterials(6). Thus, it seems attractive and beneficial to apply osteoinductive biomaterials to reconstruct bone defects of critical sizes, since new bone formation led by osteoconductive biomaterials cannot fill up most of such bone defects. Although still not fully understood, mechanisms of osteoinduction have been extrapolated to include a direct induction of recruitment, proliferation and differentiation of mesenchymal stem cells from blood, fat or muscle tissue to cells in the bone forming lineage by osteoinductive biomaterials, and an indirect induction by proteins which induce new bone formation and are absorbed to the osteoinductive biomaterials during in vivo implantations(1, 7).

Autologous bone grafts contain viable precursor cells for osteogenesis and possess excellent biological and mechanical properties for both osteoconduction and osteoinduction, thus they have been regarded as the ‘golden standard’ in reconstructing bone defects in oral and maxillofacial surgery(8). Drawbacks in using autologous bone grafts to reconstruct bone defects include limited availability of donor sites(9), morbidity in donor sites, risks of infections, nerve damages, hemorrhage, prolonged surgical procedures(10, 11) and unpredictable resorption of autologous bone grafts after implantation(12-14). These drawbacks impose necessities of using and developing bone graft substitutes with similar biological and mechanical properties as well as clinical performances. Among different bone graft substitutes, synthetic biomaterials are of especially high research interest, because they can be designed to possess bioactivities similar to autologous bone grafts and be produced in controlled manners and a massive amount. Synthetic calcium phosphate bioceramics, a prominent class of bone graft substitutes, have been widely used in trauma and orthopedic surgery in the Netherlands(15), and are considered as good alternatives for autologous bone grafts(15, 16) because they provide a source of calcium ions and
phosphate ions, which are necessary for new bone formation in bone defects, during their degradation. Hydroxyapatite (HA) bioceramics, which appears as the inorganic component in nature bone tissue, provides good mechanical support in bone defects in non-load bearing sites, have been used as an option for bone graft substitutes, and take years before a full degradation(17). HA bioceramics are reported to show osteoinductive potentials in dogs(18, 19) and baboons(20) when they are implanted subcutaneously or intramuscularly. β-tricalcium phosphate (β-TCP) bioceramics degrade faster than HA bioceramics due to their higher solubility in vivo, possess good osteoconductive capacity and serve as excellent scaffolds for bone regeneration in bone defects(21, 22). Biphasic calcium phosphate (BCP) bioceramics are mixtures of HA and β-TCP at different ratios, often 60% of HA with 40% β-TCP(23) or 80% HA with 20% β-TCP(24). BCP bioceramics in forms of particles, blocks or injectable substances turn to be highly promising bone substitutes for uses in orthopedic, dental and maxillofacial surgeries thanks to their good bioactivities derived from the combination of HA and β-TCP(25-27). Although chemical compositions of different calcium phosphate bioceramics are similar or even identical, their bioactivities differ due to differences in porosities in macro- and micro-view(15), sintering temperature(24), the ratio of calcium and phosphorus(28), particles sizes(29), overall geometry(30), surface roughness and specific surface area(31). Currently it is still to be determined what parameters make calcium phosphate bioceramics fully resemble autologous bone grafts and subsequently replace their use.

Despite excellent bioactivities and biological performances of calcium phosphate bioceramics in vitro and in vivo, their inherent high brittleness prevents their plastic deformation and hinder their wide clinical applications, especially in load bearing sites (17, 32). A feasible solution to such a problem is to incorporate calcium phosphate bioceramics into polymeric matrices, since natural bone tissue is essentially mineralized collagen matrices(33).

The presented study first aims to test osteoinductive properties of five different calcium phosphate bioceramics, with a special interest in the influence of granule sizes in osteoinduction, in a sheep model. Since BCP particles in the size range of 45-150 µm have been shown to be osteoinductive(34), the other aim of our study is to investigate whether incorporation of BCP particles and two other β-TCP particles in the same size range into PTMC matrices makes the composite scaffolds osteoinductive as well. Besides, the biodegradation and biocompatibility of all tested calcium phosphate biomaterials and PTMC-calcium phosphate composite scaffolds are studied.

MATERIALS AND METHODS

Materials

Table 1 presents the physiochemical and structural characteristics of different calcium phosphate and PTMC-calcium phosphate composite biomaterials included in our study.

BCP particles of two different size ranges, 45-150 µm (B45P) and 150-500 µm (B150P), and β-TCP particles of 45-150 µm (T45P) were provided by Xpand Biotechnology BV, Bilthoven,
the Netherlands. The BCP particles contained 20±3% β-TCP and 80±3% HA and were sintered at 1150 °C. β-TCP particles from Xpand contained 90% β-TCP and 10% HA. These particles from Xpand all possessed porous structures with pore sizes of around 1 µm, which can only been seen under electron microscope (Figure 1). The BCP particles had a microporosity of 17% and a specific surface area of 1.0 m²/g(34).

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Table 1. Overview of the included biomaterials

<table>
<thead>
<tr>
<th>Code</th>
<th>Chemical composition</th>
<th>Form and size</th>
<th>Porosity</th>
<th>Implanted volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>B45P</td>
<td>20/80 (TCP/HA)</td>
<td>BCP particles</td>
<td>Microporous</td>
<td>1 ml</td>
</tr>
<tr>
<td></td>
<td>45-150 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B150P</td>
<td>20/80 (TCP/HA)</td>
<td>BCP particles</td>
<td>Microporous</td>
<td>1 ml</td>
</tr>
<tr>
<td></td>
<td>150-500 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T45P</td>
<td>90/10 (TCP/HA)</td>
<td>β-TCP particles</td>
<td>Microporous</td>
<td>1 ml</td>
</tr>
<tr>
<td></td>
<td>45-150 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T45C</td>
<td>100% β-TCP</td>
<td>β-TCP particles</td>
<td>Non- Microporous</td>
<td>1 ml</td>
</tr>
<tr>
<td></td>
<td>45-150 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T150C</td>
<td>100% β-TCP</td>
<td>β-TCP particles</td>
<td>Non- Microporous</td>
<td>1 ml</td>
</tr>
<tr>
<td></td>
<td>150-500 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTMC-B45P</td>
<td>70/30 (PTMC/BCP)</td>
<td>Disc shape N/A</td>
<td>70% in macroview</td>
<td>Φ = 5 mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 mm in thickness</td>
</tr>
<tr>
<td>PTMC-T45P</td>
<td>70/30 (PTMC/TCP)</td>
<td>Disc shape N/A</td>
<td>70% in macroview</td>
<td>Φ = 5 mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 mm in thickness</td>
</tr>
<tr>
<td>PTMC-T45C</td>
<td>70/30 (PTMC/TCP)</td>
<td>Disc shape N/A</td>
<td>70% in macroview</td>
<td>Φ = 5 mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 mm in thickness</td>
</tr>
<tr>
<td>PTMC</td>
<td>100% PTMC</td>
<td>Disc shape N/A</td>
<td>70% in macroview</td>
<td>Φ = 5 mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 mm in thickness</td>
</tr>
</tbody>
</table>

Figure 1. Scanning electron microscope images of β-TCP particles and BCP particles in the size range of 45-150 µm from Xpand. A: β-TCP particles; B: BCP particles. Images are kindly provided by Xpand Biotechnology BV, Bilthoven.
β-TCP particles of two different size ranges, 45-150 µm (T45C) and 150-500 µm (T150C), were provided by CAM Bioceramics BV, Leiden, the Netherlands. The β-TCP particles were sintered at 1300 °C for 8 hours and did not contain microporous structures.

1,3-trimethylene carbonate of polymerization grade (Boehringer Ingelheim, Germany), stannous octoate (SnOct2, Sigma, USA), and other solvents (Biosolve, the Netherlands) of technical grade were used as received. For the salt leaching procedure to create porous scaffolds, sodium chloride (NaCl) (Merck) crystals were fractioned into a size range of 200-435 µm by being sieved through meshes of the sizes on a Fritsch sieving machine and were stored in a cool, dry place.

The synthesis and characterization of poly(trimethylene carbonate) has been described in details in a previous study(34). The synthesized PTMC polymer was purified by being dissolved in chloroform and precipitated in a five-fold excess of pure ethanol. A salt leaching technique was used to produce porous PTMC scaffolds. The purified PTMC polymer was dissolved into chloroform at a concentration of 5 g/100 ml and then the sieved NaCl crystals were dispersed into the PTMC-chloroform solution by magnetic stirring. The amount of added NaCl crystals took up 70 vol% of the PTMC fraction. Then the mixed PTMC-NaCl dispersion was precipitated in a five-fold excess of pure ethanol and the PTMC-NaCl precipitation was collected and dried under vacuum at room temperature until constant weight. Dried PTMC-NaCl precipitation was compression molded into discs of 5 mm in diameter and 5 mm in thickness at 140°C under a pressure of 3.0 MPa using a Carver model 3851-0 laboratory press (Carver, USA).

The abovementioned BCP particles from Xpand, β-TCP particles from Xpand, and β-TCP particles from CAM, all in the size range of 45 to 150 µm, were mechanically dispersed into the synthesized PTMC polymer in order to create a PTMC-calcium phosphate composite with 30 vol% (equal to 50 wt%) of calcium phosphate particles. The production of PTMC-T45C composite is taken as an example to describe the producing procedure. The T45C particles were dispersed into the PTMC-chloroform solution with a PTMC concentration of 5 g/100 ml by magnetic stirring to form a homogeneous dispersion. The same salt leaching and compression molding technique as how porous PTMC discs were created were applied to create porous PTMC-T45C composite discs. The prepared PTMC-NaCl discs and PTMC-T45C-NaCl discs were then sealed under vacuum and exposed to 25 KGy γ-irradiation from a 60Co source (Isotron BV, Ede, the Netherlands) for sterilization. During the sterilization procedure, the PTMC matrices became simultaneously cross-linked(35). To create porous structures, all discs were gently stirred in demineralized water for a period of three days under sterile conditions to wash out the added NaCl crystals. The demineralized water was changed four times a day. Porous discs of PTMC-T45P and PTMC-B45P in the same size were created and sterilized in the same methods as the porous PTMC-T45C composite discs.

**Surgical procedure**

All procedures performed on the sheep were in compliance with the international standards on animal welfare and regulations of the Animal Research Committee of University Medical Center Groningen under the project number 5611.
Ten female adult Dutch Texel sheep were included in the *in vivo* study. The abovementioned different calcium phosphate particles and PTMC-calcium phosphate composite scaffolds were implanted in the paraspinal muscles in the sheep under general anesthesia (Figure 2). The general anesthesia was induced with 20 mg/kg sodium pentothal and 2.5 ml 50 mg/ml Finadyne and maintained by inhalation of 3% sevoflurane. The implantation sites on the back were marked with non-resorbable sutures (Polypropylene, Ethicon, USA) in muscle fascia. The wounds were closed layer by layer with resorbable sutures (Polyglactin 910, Ethicon, USA). Amoxicillin (15 mg/kg) was administered before surgery and until six days after the operation. Buprenorphine was applied for pain relief before and after surgery.

Fluorochrome markers were injected to monitor potential new bone formation with time passing by. Table 2 shows the schedule of injections of fluorochromes. The sheep were sacrificed at three months and nine months, five for each time point, respectively, by an injection of overdosed pentobarbital (Organon, the Netherlands). After each termination, muscle tissue containing the implants were retrieved and fixed in a 4% phosphate buffered formalin solution.

### Figure 2. surgery scheme. A: Placing calcium phosphate particles (B45P, B150P, T45P, T45C, T150C) intramuscularly; B: Placing PTMC-calcium phosphate composite scaffolds (PTMC-B45P, PTMC-T45P, PTMC-T45C), or PTMC porous scaffolds intramuscularly. Different scaffolds are shown by the arrow(↓). Notice that none of the abovementioned biomaterials were fixated in the implantation sites.

### Table 2. Schedule of fluorochrome injection

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Solvent</th>
<th>Administration</th>
<th>Time of injection for 3-month group</th>
<th>Time of injection for 9-month group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcein</td>
<td>Sigma, the Netherlands</td>
<td>2% NaHCO₃</td>
<td>After three weeks</td>
<td>After 30 weeks</td>
</tr>
<tr>
<td>Xylenol Orange</td>
<td>Sigma, the Netherlands</td>
<td>1% NaHCO₃</td>
<td>100 mg/kg, intravenously</td>
<td>After 33 weeks</td>
</tr>
<tr>
<td>Oxytetracycline (Engemycin)</td>
<td>Mycofarm, the Netherlands</td>
<td>Physiological saline</td>
<td>After six weeks</td>
<td>After 36 weeks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-8</td>
<td>6-8</td>
<td>6-8</td>
</tr>
</tbody>
</table>
**Preparation for histological evaluation**

After being rinsed thoroughly with phosphate buffered solutions and dehydrated in gradient ethanol solutions, the fixed samples were embedded in methyl methacrylate (LTI, the Netherlands) without being decalcified. Sections of 20-30 μm in thickness for histological evaluation were sawed through the center of the samples parallel to the long axis of sheep dorsal muscle by a modified diamond saw (Leica SP1600, Leica Microsystems, Germany). 1% methylene blue (Sigma-Aldrich, the Netherlands) and 0.3% basic fuchsin (Sigma-Aldrich, the Netherlands) were used to counterstain sections for observations under light microscope. The sections were digitalized by a slide scanner (Dimage Scan Elite 5400 II, Konica Minolta Photo Imaging Inc., Japan) for observations under 25× magnification and by a digital camera on Leica microscope (DFC 420 C, Leica microsystems, Germany) for observations under 200× magnification. Histological evaluation on the sections focused on new bone formation induced by the biomaterials, degradation of the biomaterials, and tissue reactions towards the biomaterials. New bone formation induced by the biomaterials was counted by their incidence and was scored according to the amount of newly formed bone from zero to three. Zero stood for no new bone formation observed, one stood for limited new bone formation or only mineralization observed, two stood for moderate new bone formation, and three stood for abundant new bone formation. Unstained sections were produced for observations under an epifluorescent confocal laser microscope (Leica TCS SP2, Leica, Germany) to monitor the dynamics of new bone formation, if there was any. The observation was carried out under a 20× oil immersion objective. Calcein with a peak absorption wavelength (abs.) of 500 nm and a peak emission wavelength (em.) of 545 nm displayed green in the images, xylene orange of 543 nm abs. and 580 nm em. displayed red in the images, and tetracycline of 405 nm abs. and 560 nm em. displayed blue in the images.

**Histomorphometry**

Digital images obtained from the slide scanner were used for histomorphometry under Adobe Photoshop CS6. In each image, an overall region of interest (ROI) was determined to contain the implantation site and the newly formed bone, if there was any, and the corresponding pixels were registered. Then newly formed bone, if there was any, was manually selected using ‘Magic Wand Tool’ with a tolerance set as ‘50’, and the corresponding pixels were registered. The percentage of newly formed bone was calculated by the following formulation:

\[
\text{Percentage of newly formed bone} = \frac{\text{Pixels of newly formed bone}}{(\text{Pixels in ROI} - \text{Pixels of newly formed bone})} \times 100\%
\]

The calculation was performed twice with the researcher blinded from the information of the sections. The mean percentage of newly formed bone and the standard deviation were
reported in the results. A Mann-Whitney U test was performed to compare the performances of different materials.

RESULTS

One sheep, which had been healthy, died six months after the surgery and causes for the unexpected death were not revealed by a post mortem autopsy. Samples from the deceased sheep were harvested and included in the nine months follow-up group. The other sheep did not show signs of infections or other complications during the experiment. Although the implantation sites were marked by non-degradable sutures, difficulties existed in relocating and retrieving the implanted biomaterials after time spans of several months, due to that the biomaterials were biodegradable and were not fixed in the paraspinal muscle tissue. Therefore, unfortunately, a whole collection of samples containing every implanted biomaterial could not be retrieved.

Figure 3 shows an overview of histological observations of the implanted calcium phosphate bioceramic particles under 25× magnification, and figure 4 shows an overview of histological observations of PTMC-calcium phosphate composite scaffolds and PTMC scaffolds under 25× magnification. Figure 5 shows histological observations under 200× magnification concerning new bone formation, degradation and tissue reactions towards the calcium phosphate bioceramic particles. Table 3 shows the histological evaluation and histomorphometry calculation on new bone formation induced by different calcium phosphate bioceramic particles.

Histological evaluation of the sections was carried out under light microscope to distinguish whether there was new bone formation or not. Abundant new bone formation was seen induced by BCP particles of both size ranges at both three months and nine months. New bone formation was not observed in the groups of different β-TCP particles at neither time points, from neither sources, nor of different size ranges. No new bone formation occurred in the groups of PTMC-calcium phosphate composite scaffolds and PTMC scaffolds, therefore grading of new bone formation and histomorphometry were not carried out for these biomaterials.

Induced by BCP particles of both size ranges, new bone formation occurred in close contact to surfaces of the BCP particles (Figure 5 A-D). At three months new bone was formed into continuous patches outlining contours of the implantation sites and scattering small pieces interconnecting remaining BCP particles. At nine months newly formed bone was observed to be of larger amount and more mature with clearly visible Haversian system than at three months. New bone induced by the BCP particles resembled cancellous bone in structure at nine months. As for the amount of newly formed bone, no statistical significance was shown between the BCP particles of both size ranges, regardless of the two time points.

Figure 6 shows observations of new bone formation induced by the BCP particles of both size ranges under epifluorescent confocal microscopy. Different colors from fluorochrome markers represented the deposition of newly formed bone in a time order. New bone
formation started as early as three weeks after the implantation of the BCP particles and continued until the animals were sacrificed. New bone formation and bone remodeling still remained active at nine months.

At three months, disintegration of BCP particles and TCP particles was observed to different extents. Most of the BCP particles of 45-150 µm remained intact with reduced sizes and these intact particles were surrounded by “dust like” disintegrated particles (Figure 3 A). BCP particles of 150-500 µm showed reduced size and remained intact. Areas containing the “dust like” disintegrated BCP particles were not seen in the implantation site (Figure 3 C). Compared to BCP particles of both size ranges, β-TCP particles showed advanced disintegration. β-TCP particles of 45-150 µm from Xpand have been obviously disintegrated into clusters of fine TCP particles. Only scarce T45P particles remained intact with a reduced size (Figure 3 E). β-TCP particles from CAM bioceramics of both size ranges have been substantially disintegrated and the whole implantation site got filled with fine TCP particles (Figure 3 G, I). Only few TCP granule of 150-500 µm from CAM bioceramics were seen intact with a reduced size (Figure 3 I).

**Table 3.** New bone formation induced by different calcium phosphate bioceramic particles

<table>
<thead>
<tr>
<th>Code</th>
<th>Time point</th>
<th>Incidence</th>
<th>Amount (average score)</th>
<th>Histomorphometry calculation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B45P</td>
<td>3 months</td>
<td>2/4</td>
<td>1</td>
<td>3.42±4.62</td>
</tr>
<tr>
<td></td>
<td>9 months</td>
<td>3/5</td>
<td>1.8</td>
<td>7.31±2.12</td>
</tr>
<tr>
<td>B150P</td>
<td>3 months</td>
<td>3/5</td>
<td>1.2</td>
<td>2.99±4.85</td>
</tr>
<tr>
<td></td>
<td>9 months</td>
<td>3/5</td>
<td>2</td>
<td>9.03±2.46</td>
</tr>
<tr>
<td>T45P</td>
<td>3 months</td>
<td>0/3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9 months</td>
<td>0/4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T45C</td>
<td>3 months</td>
<td>0/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9 months</td>
<td>0/3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T150C</td>
<td>3 months</td>
<td>0/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9 months</td>
<td>0/4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
At three months, PTMC matrices have been degraded into remnants of different sizes in PTMC-calcium phosphate composite scaffolds and PTMC scaffolds (Figure 4 a, c, e, g and Figure 7 3m). The implantation sites of PTMC-B45P were filled with fine BCP particles disintegrated from B45P particles and some intact B45P particles with reduced size were present. A few remaining B45P particles were stained red on the sections, implying that certain protein absorption occurred on the microporous surfaces of the B45P particles despite of no new bone formation observed in these samples (Figure 4 a). Fine TCP particles disintegrated from T45P particles were not seen in the implantation site of PTMC-T45P and only a few intact T45P particles with reduces size remained. A few red-stained remaining T45P particles were also present in the implantation sites (Figure 4 c). T45C particles were all disintegrated into fine TCP particles and degradation of these fine T45C particles has already progressed much. Very few fine TCP particles scattered in the implantation site of PTMC-T45C (Figure 4 e).

At nine months, degradation of all implanted biomaterials has progressed further. Intact BCP particles of both size ranges with reduced sizes were still present in the implantation sites, but the number of these remaining BCP particles has been much reduced compare to at three months. The ‘dust like’ areas consisting of disintegrated BCP particles in the implantation sites of B150P particles were not as prominent as B45P particles (Figure 3 B, D). β-TCP particles of both size ranges from both sources have been almost completely disintegrated (Figure 3 F, H, J). Only few countable T45P particles with much reduced size were spotted in the implantation sites (Figure 3 F). No intact β-TCP particles of both size ranges from CAM but only clusters of fine β-TCP particles were left in the implantation sites (Figure 3 H, J). Few intact BCP particles with much reduced size were still present in the implantation site of PTMC-B45P (Figure 4 b). TCP particles of 45-150 µm from both sources went through a complete disintegration and degradation and disappeared from the implantation sites of PTMC-T45P and PTMC-T45C scaffolds (Figure 4 d, f). PTMC matrices of the PTMC-calcium phosphate composite scaffolds have been degraded into homogenous small remnants, which were being processed by macrophages and foreign body giant cells (Figure 7 9m). Interestingly, degradation of PTMC scaffolds at nine months resulted in
OSTEOINDUCTIVITY OF CAP BIOMATERIAL AND PTMC-BCP COMPOSITES

PTMC-B45P

PTMC-T45P

PTMC-T45C

PTMC

1 mm

1 mm

1 mm
Figure 5. Observations under 200× light microscope of different calcium phosphate bioceramic particles implanted intramuscularly after three months and nine months. New bone induced by BCP particles of both 45-150 µm and 150-500 µm was formed in close contact to the BCP particles at both three months and nine months. b: newly formed bone; HS: Haversian system; g: calcium phosphate particles; g': red-stained calcium phosphate particles; *: disintegrated calcium phosphate particles; c: fibrous connective tissue; a: adipose tissue. Scale bar represents 50 µm.
Figure 6. Observations of BCP particles of 45-150µm (A-D) and 150-500 µm (E-H) implanted intramuscularly under epifluorescent confocal microscopy after three months (A, B, E, F) and nine months (C, D, G, H). Images A, C, E and G are observations under light field, while images B, D, F and H are corresponding epifluorescent confocal images. New bone formation started as early as three weeks after implantation and continued through the whole implantation time spans. New bone formation and bone remodeling remained active at nine months. CG: calcein, green; XO: xylene orange, red; TC: tetracycline, blue. Scale bar represent 200µm.
Figure 7. Observations under 200× light microscope of different PTMC-calcium phosphate composite scaffolds implanted intramuscularly after three months and nine months. * PTMC remnants; g: calcium phosphate particles; arrows point at foreign body giant cells. Scale bars represent 50 µm.

discernable round voids surrounded by layers of fibrous tissue (Figure 4h), which could possibly be explained by a halted degradation due to the decrease of molecular weight during their degradation procedure(36).

The host tissue executed a homogenous reaction towards all implanted biomaterials (Figure 4, 5). Excessive loose fibrous tissue encapsulated, infiltrated, and segregated the implantation sites at three and nine months. Inflammatory cells, such as macrophages and foreign body giant cells, were presented in the implantation sites, processing disintegrated calcium phosphate particles and degraded PTMC matrices. At nine months, the implantation sites were largely replaced by adipose tissue.
DISCUSSION

This study was carried out to assess osteoinductive properties of different calcium phosphate bioceramic particles and PTMC-calcium phosphate composite scaffolds and to evaluate the in vivo degradation characteristics and tissue reactions towards these biomaterials.

Biomaterials with osteoinductive properties have been shown to enhance their regenerative performances in critical orthotopic bone defects(6), thus research on osteoinductive biomaterials becomes an increasingly hot topic. Whether a biomaterial is osteoinductive or not is determined by various factors(7). Crucial determining factors of a ceramic biomaterial being osteoinductive include chemical composition, that is the ratio between β-TCP and HA(28); grain size(29); synthesizing temperature(24); porosity under macroview and microview(15); surface roughness and specific surface area(31).

BCP bio ceramics have been proven to possess osteoinductive capacities(6, 24) thanks to a balance between the presence of β-TCP, which is more resorbable, and HA, which is more stable and resorption-resistance(20, 27). Implanting β-TCP particles from Xpand, which contained 10% of HA, intramuscularly in sheep led to no new bone formation, indicating the important role of HA in making bioceramics osteoinductive. Mechanical stability of ceramic biomaterials also influences their capacity of inducing new bone formation(26). The β-TCP particles from both sources disintegrated too fast to provide a stable surface for new bone formation, thus low mechanical stability added to their incapability of osteoinduction, besides the lack of HA in their composition.

In our study, BCP particles of two different size ranges were revealed to induce substantial new bone formation in sheep long dorsal muscle. A previous study showed that BCP particles of 45-150 µm were osteoinductive(34). Compared to the previous study, BCP particles of a larger size range showed similar osteoinductive capacity. Mixture of HA and β-TCP particles (Zimmer, Warsaw, IN) ranging from less than 44 µm up to 1000 - 2000 µm were seeded with human bone marrow stromal cells and implanted in mice subcutaneously. Mixed HA/TCP particles in a size range of 100 - 500 µm led to the most abundant new bone formation, indicating HA/TCP particles in a size range of 100-500 µm provides highly suitable niches for human bone marrow stromal cells to attach, infiltrate, proliferate and differentiate(37), echoing the finding in our study. Besides, BCP particles with a HA/TCP ratio of 60:40 and a size range of 1000 to 2000 µm have been shown to induce mature de novo bone which bridges remaining BCP particles and resembles natural cancellous bone after six months of implantation in sheep back muscles(29). Therefore, granule sizes of a wide range influence osteoinductive properties of biomaterials.

Synthesizing temperature strongly influences the microporosity, surface roughness and specific surface area of a ceramic biomaterial. BCP particles and HA particles synthesized at relatively lower temperatures possess fine microporous structures within the macropore walls and their specific surface area increases with the decrease of synthesizing temperature(38). A microporous structure in ceramic biomaterials facilitates diffusion of body fluid through the bioceramics and thus provide a better environment for cells to infiltrate and
differentiate. BCP particles synthesized at 1150°C enhance osteogenic differentiation of human multipotent marrow stromal cells\(^{(39)}\) and BCP scaffolds produced at 1150°C lead to new bone formation throughout the whole constructs both in iliac wing defects and in intramuscular implantation in goats\(^{(6)}\). The BCP particles tested in our study were sintered at 1150°C for 8 hours and resulted in potent osteoinductive capacity. One possible explanation for why TCP particles from CAM Bioceramics sintered at 1300°C did not induce new bone formation intramuscularly, aside from not containing any HA, is the relatively high sintering temperature, which leads to a decrease in their microporosity and specific surface areas. In our study, some BCP particles and TCP particles from Xpand were observed to be stained red in the sections, implying that protein absorption occurred on the rough surfaces of their microporous structure despite of no new bone formation happening around those particles.

None of the PTMC-calcium phosphate composite scaffolds tested in our study induced new bone formation in the sheep long dorsal muscle. Given the 70% of porosity in the PTMC-calcium phosphate composite scaffolds, the content of calcium phosphate bioceramic particles in the composite scaffolds is only 30% of the 30% solid material (9%) and the calcium phosphate particles, being dispersed in a continuous phase of PTMC matrix, disintegrated fast and provided low mechanical stability for new bone formation. Thus, it is not surprising that the tested PTMC-calcium phosphate composite scaffolds are not osteoinductive. A previous study shows that new bone formation of a limited amount is observed in the center of non-porous PTMC-BCP composite sheets, after the composite sheets are implanted in sheep long dorsal muscles for three months\(^{(34)}\). The PTMC-BCP composite sheets contain 30% BCP particles of 45-150 μm and are of 1.5 mm thick. Compared to our PTMC-BCP porous scaffolds, the PTMC-BCP sheets contain more BCP particles and are thinner. Since PTMC matrices are degraded through surface erosion, such non-porous composite sheets offer more chances for BCP particles to be released into the surrounding tissue and exert their osteoinductivity. Le Nihouannen et al. implant BCP/fibrin composites of two to three cm\(^3\) in sheep long dorsal muscles for six months. The implanted BCP/fibrin composites contain BCP particles with a HA/β-TCP ratio of 60/40 and a diameter of one to two mm and a fibrin glue of 4 IU. Formation of well mineralized, matured bone tissue bridging the BCP particles has been confirmed by histology, back scattered electron micrographs and μCT. The number and thickness of the formed bone trabeculae are similar to those in vertebral body\(^{(40)}\).

**CONCLUSION**

Biphasic calcium phosphate particles of 45-500 μm induced abundant *de novo* bone tissue after being implanted in sheep dorsal muscle for three and nine months and were potently osteoinductive. Porous composite scaffolds composed of PTMC matrices and three different β-tricalcium phosphate particles of 45-150 μm induced no new bone formation in sheep dorsal muscle during the same implantation periods and showed no osteoinductive capacities. Implantation of different calcium phosphate bioceramic particles of different sizes and PTMC-calcium phosphate composite scaffolds in sheep long dorsal muscle led
to uneventful degradation of the abovementioned biomaterials and provoked no serious tissue reaction. Future studies are needed to determine optimal compositions of composite biomaterials based on PTMC and calcium phosphate to produce osteoinductive composites.
REFERENCES


