Evaluation of the biocompatibility of calcium phosphate cement/PLGA microparticle composites

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Received 16 April 2007; revised 3 September 2007; accepted 18 October 2007
Published online 15 January 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.31831

Abstract: In this study, the biocompatibility of a calcium phosphate (CaP) cement incorporating poly (D,L-lactic-co-glycolic acid) (PLGA) microparticles was evaluated in a subcutaneous implantation model in rats. Short-term biocompatibility was assessed using pure CaP discs and CaP discs incorporating PLGA microparticles (20% w/w) with and without preincubation in water. Long-term biocompatibility was assessed using CaP discs incorporating varying amounts (5, 10, or 20% w/w) and diameter sizes (small, 0–50 μm; medium, 51–100 μm, or large, 101–200 μm) of PLGA microparticles. The short-term biocompatibility results showed a mild tissue response for all implant formulations, irrespective of disc preincubation, during the early implantation periods up to 12 days. Quantitative histological evaluation revealed that the different implant formulations induced the formation of similar fibrous tissue capsules and interfaces. The results concerning long-term biocompatibility showed that all implants were surrounded by a thin connective tissue capsule (<10 layers of fibroblasts). Additionally, no significant differences in capsule and interface scores were observed between the different implant formulations. The implants containing 20% PLGA with medium- and large-sized microparticles showed fibrous tissue ingrowth throughout the implants, indicating PLGA degradation and interconnectivity of the pores. The results demonstrate that CaP/PLGA composites evoke a minimal inflammatory response. The implants containing 20% PLGA with medium- and large-sized microparticles showed fibrous tissue ingrowth after 12- and 24-weeks indicating PLGA degradation and interconnectivity of the pores. Therefore, CaP/PLGA composites can be regarded as biocompatible biomaterials with potential for bone tissue engineering and advantageous possibilities of the microparticles regarding material porosity. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 87A: 760–769, 2008

Key words: CaP/PLGA cement; biocompatibility; interconnectivity

INTRODUCTION

The filling of bone defects resulting from trauma or surgical resection of tumors requires the availability of safe and reliable synthetic bone substitutes, since obtaining autologous bone is accompanied with the known disadvantages of donor site morbidity, increased operative time, and insufficient volumes. Calcium phosphate (CaP) cements have been proven to be useful as bone substitutes at nonload bearing sites. Previous work on CaP cement has shown that if it consists of a mixture of powder and liquid, this CaP cement becomes injectable, and hence can be shaped perfectly according to the defect dimension in situ. Porosity can be created by incorporating degradable poly (D,L-lactic-co-glycolic acid) (PLGA) microparticles in the CaP cement to enhance tissue ingrowth in the CaP cement. PLGA microparticles will be hydrolyzed in vivo and as a consequence create microporosity. PLGA polymers are interesting due to degradation properties, which can be tailored by changing molecular weight, tacticity, and lactic to glycolic ratio. Although a previous study with various small-sized CaP/PLGA composite implants (0, 15, 30, or 50% w/w PLGA microparticles) showed an excellent bone response, additional studies to the biocompatibility of CaP/PLGA implants have to be performed to exclude that the incorporated PLGA microparticles evoke an inflammatory response during their degradation. In the design of such an experiment, it has to be
included that PLGA microparticle size can affect the foreign body reaction at the surface of the microparticles. Also, by varying the amounts and diameter sizes of PLGA microparticles a threshold for interconnectivity can be determined. Furthermore, these PLGA microparticles can be loaded with growth factors to stimulate bone formation and cement resorption in future applications.

Also, the biocompatibility of CaP cement could be improved by preincubation in water. Previous research resulted in a better cellular response after preincubation in vitro, which could also have an effect on the final in vivo tissue response.

In view of the above mentioned, this study focused at the biocompatibility of CaP cement incorporating PLGA microparticles in a subcutaneous implantation model in rats. A subcutaneous location was proven before to be an appropriate location to evaluate the inflammatory reaction associated with CaP cement. Short-term biocompatibility was assessed using CaP discs and CaP discs incorporating PLGA microparticles (20% w/w) with and without preincubation in water. Long-term biocompatibility was assessed using CaP discs incorporating various amounts (5, 10, or 20% w/w) and diameter sizes (small, 0–50 μm; medium, 51–100 μm, or large, 101–200 μm) of PLGA microparticles.

### MATERIALS AND METHODS

#### Substrates

The CaP cement Calcibon® (Merck Biomaterial GmbH, Darmstadt, Germany) was used, which has a chemical composition of 61% α-TCP, 26% CaHPO₄, 10% CaCO₃, and 3% PHA (α-TCP is alpha tri-calcium phosphate, PHA is precipitated hydroxyapatite). An aqueous solution of 1% Na₂HPO₄ was used as the liquid component. The used liquid/powder ratio was 0.35 mL/g. Before usage, the cement powder was sterilized by γ-radiation with 25 kGy (Isotron B.V., Ede, The Netherlands) The cement liquid was filter-sterilized through a sterile 0.2-μm filter.

#### PLGA microparticles

PLGA (Purasorb®, Purac, Gorinchem, The Netherlands) with a molecular weight of 48 kDa and a lactic to glycolic acid ratio of 50:50 was used. PLGA microparticles were prepared using a (water/oil/water) double emulsion solvent evaporation technique. The microparticles were produced by dissolving 1.0 g PLGA in 4 mL of dichloromethane (DCM) inside a glass tube. After dissolution, 500 μL deionized water was added to this mixture and emulsified for 60 s on a vortexer. Subsequently, 6 mL 0.3% aqueous polyvinyl alcohol (PVA) solution was added and vortexed for another 60 s to produce the second emulsion. The suspension was stirred for 1 h. After stirring, the microparticles were allowed to settle for 15 min and the solution was decanted. The microparticle suspension was centrifuged, and the clear solution at the top was decanted. Then 5 mL of deionized water was added, the microparticles were washed, centrifuged and the solution was aspirated. Finally, the microparticles were frozen, freeze-dried for 24 h and stored under argon at 20±8°C.

For the short-term biocompatibility study, PLGA microparticles ranging from 5 to 200 μm were used with an average diameter of 33 μm. For the long-term biocompatibility study, microparticles were sieved with a sieve shaker (Retsch GmbH, Haan, Germany) with 50, 100, and 200 μm diameter sieves to obtain batches of small (0–50 μm), medium (51–100 μm), and large (101–200 μm) PLGA microparticles.

#### Material and methods

**TABLE I**

<table>
<thead>
<tr>
<th>Method</th>
<th>PLGA Amount</th>
<th>Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Short-term Biocompatibility (2,4,8 and 12 days)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaP0</td>
<td>—</td>
<td>0–200</td>
</tr>
<tr>
<td>CaP0/PLGA 20%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaP8</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>CaP8/PLGA 20%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Long-term Biocompatibility (12 and 24 weeks)</strong></td>
<td>5%</td>
<td>0–50</td>
</tr>
<tr>
<td>CaP0/PLGA 5%</td>
<td></td>
<td>51–100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>101–200</td>
</tr>
<tr>
<td>CaP0/PLGA 10%</td>
<td></td>
<td>0–50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51–100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>101–200</td>
</tr>
<tr>
<td>CaP0/PLGA 20%</td>
<td></td>
<td>0–50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51–100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>101–200</td>
</tr>
</tbody>
</table>

**Materials and Methods**

**Table 1.** Overview of the CaP/PLGA Disc Formulations

Figure 1. Schematic drawing of a CaP/PLGA implant showing the four predetermined areas used in the histological evaluation.

The discs were prepared by adding 350 μL cement liquid (1% w/w aqueous solution of Na₂HPO₄) to a 1000 mg CaP cement powder or CaP/PLGA mixture in a 2 mL syringe (Becton Dickinson, Alphen a/d Rijn, The Netherlands). The syringe was closed with an injection plunger and placed in a commercially available mechanical mixing apparatus as used for the preparation of dental amalgam.

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**Journal of Biomedical Materials Research Part A**
and dental cements (Silamat, Ivoclar Vivadent AG, Schaan, Liechtenstein). After mixing for 15 s, the plunger was removed and the cement/composite was injected in teflon moulds to ensure a standardized shape of the specimens. The discs (2.4 mm height and 6 mm diameter) were removed from the moulds after setting of the cement at room temperature. Discs were used without (CaP0) and with (CaP8) preincubation in water (8 weeks immersion on a rotating plate with water, refreshing three times a week at room temperature).

**CaP/PLGA microparticle composites**

Various CaP/PLGA composites were created by adding PLGA microparticles to the CaP cement. CaP cement composites with 5, 10, or 20% w/w PLGA and small, medium, or large microparticle diameter sizes were prepared (Table I). The total porosity of the different CaP/PLGA composites was determined by correlating the weight of CaP discs with the weight of CaP/PLGA discs after placement of the samples in a furnace at 650°C for 2 h to remove the PLGA microparticles.16 Afterwards, samples were analyzed using microcomputed tomography (μCT) (SkyScan 1172 high-resolution micro-CT, SkyScan, Kontich, Belgium). High-resolution scanning was performed at energy of 100 kV and intensity of 98 μA. Cone beam reconstruction (version 2.15, SkyScan) was done. Also, the chemical composition of the samples was evaluated by crushing the samples with a mortar, until the discs were reduced to powder. The powder was then analyzed by using X-ray diffraction using a θ–2θ diffractometer (XRD, Philips PW3710, Eindhoven, the Netherlands) using a CuKα radiation of 1.5418 Å wavelength. The positions and intensities of the XRD peaks were used to identify the underlying structure (phase) of the samples.

**Implantation**

Fifty-two male Wistar rats (100–120 g) were used for subcutaneous implantation. The rats received four implants each, two on each side of the vertical column. Short-term biocompatibility was assessed using four different implants (n = 6) with variation in implant composition and preincubation (Table I), and implantation periods of 2, 4, 8, and 12 days. Long-term biocompatibility was assessed using nine different implants (n = 6) with variation in PLGA microparticle amount and size (Table I), and implantation periods of 12 and 24 weeks.

Surgery was performed under general inhalation anesthesia with a combination of isoflurane, nitrous oxide, and oxygen. For reasons of reliability, implant randomization was applied regarding location. To insert the implants, the

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**Table II**

**Histological Grading Scale for Soft-Tissue Implants**

<table>
<thead>
<tr>
<th>Evaluation</th>
<th>Response</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsule quantitatively</td>
<td>1–4 fibroblasts</td>
<td>4</td>
</tr>
<tr>
<td>5–9 fibroblasts</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>10–40 fibroblasts</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>&gt;40 fibroblasts</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Not applicable</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Capsule qualitatively</td>
<td>Capsule is fibrous, mature, not dense, resembling connective or fat tissue in the noninjured regions</td>
<td>4</td>
</tr>
<tr>
<td>Capsule tissue is fibrous but immature, showing fibroblasts and little collagen</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Capsule tissue is granulous and dense, containing both fibroblasts and many inflammatory cells</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Capsule consists of masses of inflammatory cells with little or no signs of connective tissue organization</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cannot be evaluated because of infection or other factors not necessarily related to the material</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Interface qualitatively</td>
<td>Fibroblasts contact the implant surface without the presence of macrophages or leucocytes</td>
<td>4</td>
</tr>
<tr>
<td>Scattered focal of macrophages and leucocytes are present</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>One layer of macrophages and leucocytes are present</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Multiple layers of macrophages and leucocytes present</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cannot be evaluated because of infection or other factors not necessarily related to the material</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 2.** Microporosity (in %) of the different implant formulations. The 20% PLGA 0–200 μm sizes were used for the early implantation periods (short-term biocompatibility) (CaP0/PLGA and CaP8/PLGA). All other formulations were used for 12- and 24-weeks implantation (long-term biocompatibility).
animals were immobilized and placed in a ventral position. The back of the animals was shaved, washed, and disinfected with povidone-iodine. Subsequently, four small longitudinal incisions (two on each site of the vertebral column) were made. Lateral to each incision, a subcutaneous pocket was created using blunt dissection. After placement of the implants, the skin was closed using staples (Agraven™, InstruVet BV, Cuijk, the Netherlands). At the end of each implantation period, the rats were sacrificed using CO₂-suffocation.

Histology

Implants with surrounding tissue were retrieved and prepared for histological evaluation. The samples were fixed in 4% formalin solution (pH 7.4), dehydrated in a graded series of ethanol and embedded in methylmethacrylate. Following polymerization, 10-μm thick sections were prepared using a sawing microtome technique. The sections were stained with methylene blue and basic fuchsin and investigated with a light microscope to examine the amount of tissue inflammation or bone formation. The quantitative evaluation of the fibrous capsule was done in four predetermined fields using a histological grading scale for soft-tissue implants (Fig. 1 and Table II). The presented data represent the average of these four measurements.

Figure 3. X-ray diffraction (XRD) patterns of the calcium phosphate cement discs. The XRD powder patterns of as-prepared (CaP0) and preincubated discs (CaP8), both before and after subcutaneous implantation for 12 days. (* = apatite; # = β-tri calcium phosphate (TCP); + = α-TCP).

Figure 4. Microcomputed tomography (μCT) images of CaP implants containing 5% (A), 10% (B), 20% (C) PLGA 101–200 μm sizes. Together with 20% PLGA 0–200 μm sizes (D) used for the early implantation periods (short-term biocompatibility). PLGA microparticles appear as pores in the CaP cement. The size of each sample is 0.5 × 0.5 × 0.5 mm³.
Statistical analyses

Statistical analyses were performed with GraphPad Instat 3.05 software (GraphPad Software, San Diego, CA) using a one-way analysis of variance (ANOVA) with a post-hoc Tukey multiple comparisons test.

RESULTS

Implant characterization

The microporosity of the various implant formulations varied between 6.5% and 57.1%, showing lowest values for implants containing 5% PLGA small-sized and highest values for implants containing 20% PLGA large-sized microparticles (Fig. 2). XRD analysis demonstrated that the CaP0 discs were mainly composed of \( \alpha \)-tricalcium phosphate (\( \alpha \)-TCP). Preincubation of the discs for eight weeks in water (CaP8) resulted in the hydrolysis of \( \alpha \)-TCP into an apatite-like structure with some \( \beta \)-TCP. After implantation, the structure of the CaP8 samples remained the same, whereas the CaP0 samples underwent hydrolysis of \( \alpha \)-TCP into an apatite-like structure without \( \beta \)-TCP formation (Fig. 3). The incorporation of PLGA microparticles did not induce alterations of XRD patterns compared to pure CaP samples.

Microcomputed tomography (\( \mu \)CT) indicated interconnectivity within all samples containing 20% (w/w) PLGA, except for those with small diameter (0–50 \( \mu \)m) microparticles (Fig. 4).

In vivo studies

All 52 rats in this experiment remained in good health and did not show any wound complications. At retrieval, the 12- and 24-week implants were surrounded by a macroscopically visible fibrous capsule. All implants had retained their structural integrity, and no inflammatory signs or adverse tissue reactions were observed.

Short-term biocompatibility

Light microscopic analysis of the histological sections revealed that a fibrous tissue capsule developed around all implants in time. Furthermore,
inflammatory cells were present during all implantation periods (2, 4, 8, and 12 days), but amounts were declining in time. No multinucleated cells were observed within any of the different implant formulations (CaP0, CaP0/PLGA, CaP8, and CaP8/PLGA). CaP/PLGA cements, whether or not preincubated in water, did not contain fibrous tissue throughout the implants, but only concavities on the surface of the implants were filled with fibrous tissue.

After 2 days of implantation, inflammatory cells were observed in the implant vicinity without a connective tissue capsule around any of the implants. After 4 days [Fig. 5(A,B)], a thin connective tissue capsule had developed, containing inflammatory cells with some fibroblasts and a small amount of collagen bundles. After 8 days, the amount of collagen and number of fibroblasts increased in the thin connective tissue capsule and still few inflammatory cells were present. At 12 days [Fig. 5(C,D)], the thin fibrous capsule had not increased in size and the amount of fibroblasts and inflammatory cells appeared to be similar as for the 8-days implants.

Quantitative histological evaluation (Fig. 6) revealed that no significant differences in capsule and interface scores were present between the different formulations of implants (CaP0, CaP0/PLGA, CaP8, and CaP8/PLGA) at individual time points. On the other hand, capsule quality scores of the CaP0 and CaP8/PLGA group were significantly different between 4 and 12 days, both showing the transformation of a granulous and dense capsule containing fibroblasts and inflammatory cells into a mature capsule resembling connective tissue [Fig. 6(A)]. Furthermore, capsule quantity scores of the CaP8/PLGA group between 4 and 8 days were significantly different, showing an increase in the number of fibroblast layers from 1 to 4 at day 4 to 5 to 9 fibroblast layers at day 8 [Fig. 6(B)]. Finally, capsule interface scores were significantly different between days 4 and 12 in the CaP0/PLGA group, where inflammatory cells contacting the surface of the implants were replaced by fibroblasts [Fig. 6(C)].

Long-term biocompatibility

All implant formulations (CaP with 5, 10 or 20% w/w PLGA and small, medium, or large microparticle sizes) were surrounded by a connective tissue capsule containing fibroblasts, collagen bundles and few inflammatory cells after 12 weeks of implantation (Fig. 7). No multinucleated cells were observed within any of the different implant formulations. Similar observations were made after 24 weeks of implantation. Furthermore, only few inflammatory cells were present in the pores of the implants,
which were created after hydrolysis of PLGA microparticles. The implants containing 20% PLGA with medium- and large-sized microparticles showed fibrous tissue ingrowth throughout the implants [Fig. 7(C,D)], indicating PLGA degradation and interconnectivity of the pores. This is in contrast to all 5% and 10% PLGA formulations, and 20% PLGA formulations with small-sized microparticles, which only showed fibrous tissue ingrowth at the implant periphery. Quantitative histological evaluation revealed that no significant differences in capsule and interface scores were present between the different implant formulations (CaP with 5, 10, or 20% w/w PLGA and small, medium, or large microparticle sizes) at individual time points (Fig. 8).

DISCUSSION

This biocompatibility study was performed to exclude that the incorporated PLGA microparticles evoked an inflammatory response. Therefore, the short and long-term biocompatibility of CaP/PLGA formulations was examined using a subcutaneous implantation model in rats.

Physicochemical analysis demonstrated that due to implantation or preincubation in water, the α-TCP component in the CaP cement undergoes hydrolysis resulting in transformation into an apatite-like structure. After implantation, CaP cement hydrolysis was more pronounced in preincubated samples compared to nonpreincubated samples. This might be due to a longer exposure time (8 weeks) of preincubated samples to water, the difference between body fluids and only water, or a combination of both.

Histological and histomorphometrical evaluation showed only a mild tissue response to any of the different CaP/PLGA composites formulations in the early implantation periods (2, 4, 8, and 12 days). No differences in vivo were observed between pure CaP cement and CaP cement incorporating PLGA microparticles, whether or not preincubated in water. Implant preincubation was based on the results of a previous in vitro study, which showed that without
preincubation in water, CaP cement was not physiocochemically stable. This resulted in the release of ionic species, which caused cell death. The preincubation of CaP cement resulted in the hydrolysis of α-TCP into an apatite-like structure, which favored the biological response. However, the preincubated CaP cement still was not cytocompatible \textit{in vitro}.\textsuperscript{13} Nevertheless, the \textit{in vivo} results of the current study did not indicate a detrimental effect of formulations without preincubation on cells/tissues. Apparently, the capacity to buffer ionic species \textit{in vivo} is higher than in \textit{in vitro} experiments. Although PLGA is regarded as a nontoxic and biocompatible material,\textsuperscript{19} it is known that PLGA microparticles larger than 10 μm in diameter can evoke a foreign body reaction at the surface of these microparticles.\textsuperscript{11,20} These data do not confirm the results of the current study, as no inflammatory response to PLGA microparticles was observed with various (1–200 μm).

The microporosity in the CaP cement was created by incorporation and subsequent \textit{in vivo} hydrolysis of PLGA microparticles. However, incorporation of microparticles is not a guarantee to achieve interconnectivity in the composites. In the present study, fibrous tissue ingrowth after 24 weeks throughout the implant indicated complete PLGA degradation and interconnectivity in the formulations containing 20% PLGA with medium and large microparticles. Recently, Habraken et al. showed that \textit{in vitro} degradation of PLGA microparticles started after 6 weeks and was completed after 12 weeks. However, the reduction in molecular weight of the PLGA already started at day 0 and was 80% at 6 weeks when mass loss was visible.\textsuperscript{16} Therefore, the PLGA microparticles encapsulated in the CaP cement maintained their structure until 6 weeks, although molecular weight loss already commenced. In the current study, complete degradation of PLGA microparticles could only be evidenced by fibrous tissue ingrowth throughout the implants after 12 and 24 weeks.

Interestingly, in the implants containing 10% w/w PLGA with large microparticles, no fibrous tissue inside the implants was observed. This might indicate that within this CaP/PLGA formulation the
micropores were not interconnected. However, the porosity of 50.1% ± 0.7% in these implants was higher compared to the 36.2% ± 1.9% porosity of in the implants with 20% PLGA with medium microparticles. This suggests that interconnectivity does not depend solely on larger particles, but also on smaller particles as a connecting agent. The most ideal might be a mixture of smaller and larger microparticles, where smaller particles can act as a connecting agent between the larger particles to create an interconnected network. The interconnectivity in the composites with 20% PLGA with medium and large microparticles corroborates other studies indicating the necessity of pores of at least 50 μm to obtain optimal porosity of porous ceramics. Microcomputed tomography also indicated interconnectivity between the samples of 20% PLGA with medium and large sized microparticles. By applying a threshold to the images, the CaP cement was extracted form the original image. Because of reasons of arbitrary threshold as a result of alternating ranges and intensities of individual sample grey scales, μCT was not applicable for quantification of porosity and interconnectivity. Therefore, μCT was only used to visualize the appearance of the samples.

Remarkably, a study by Ruhé et al. indicated that CaP cement possesses a certain degree of intrinsic osteoinductive properties, as they found mineralized extracellular matrix in pores after PLGA degradation. However, that study consisted of smaller CaP/PLGA implants (2.0 mm height and 3.5 mm diameter) together with larger PLGA microparticles (73 ± 27 μm) combined with CO2 induced macropores, which together gave a heterogeneous distribution of macropores. Although, the current study was not designed to examine possible osteoinductivity of CaP cement, none of the composite formulations showed any formation of bone upon subcutaneous implantation. However, these observations do not exclude osteoinductive properties of CaP cement, since factors like species, implant location, and implant size are known to influence the formation of bone tissue at ectopic sites.

**CONCLUSIONS**

The results of the present study demonstrate that CaP/PLGA composites evoke a minimal inflammatory response. The implants containing 20% PLGA with medium- and large-sized microparticles showed fibrous tissue ingrowth throughout the implants after 12 and 24 weeks indicating PLGA degradation and interconnectivity of the pores. Therefore, CaP/PLGA composites can be regarded as biocompatible biomaterials with potential for bone tissue engineering and advantageous possibilities of the microparticles regarding material porosity.

We would like to thank the Dutch Technology Foundation (STW) applied science division of NWO.

**References**


