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

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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 fibro-

blasts). 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.^{1–4} 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*.^{5–8} 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.^{9,10} 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.¹¹ 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,¹² 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

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TABLE I
Overview of the CaP/PLGA Disc Formulations

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

included that PLGA microparticle size can affect the foreign body reaction at the surface of the microparticles.¹³ 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.^{14,15}

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*,¹¹ 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.⁷ 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 solving 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 poly vinyl alcohol (PVA) solution was added and vortexed for another 60 s to produce the second emulsion. After vortexing, the content of the glass tube was transferred to a 1000 mL beaker and another 394 mL of 0.3% PVA was added slowly under continuous stirring. This was directly followed by adding 400 mL of a 2% isopropyl alcohol (IPA) solution. 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°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.

Pre-set sample preparation

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

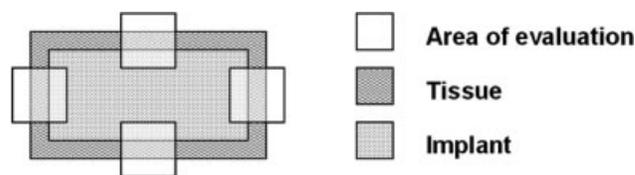


Figure 1. Schematic drawing of a CaP/PLGA implant showing the four predetermined areas used in the histological evaluation.

TABLE II
Histological Grading Scale for Soft-Tissue Implants

Evaluation	Response	Score
Capsule quantitatively	1–4 fibroblasts	4
	5–9 fibroblasts	3
	10–30 fibroblasts	2
	>30 fibroblasts	1
	Not applicable	0
Capsule qualitatively	Capsule is fibrous, mature, not dense, resembling connective or fat tissue in the noninjured regions	4
	Capsule tissue is fibrous but immature, showing fibroblasts and little collagen	3
	Capsule tissue is granulous and dense, containing both fibroblasts and many inflammatory cells	2
	Capsule consists of masses of inflammatory cells with little or no signs of connective tissue organization	1
	Cannot be evaluated because of infection or other factors not necessarily related to the material	0
Interface qualitatively	Fibroblasts contact the implant surface without the presence of macrophages or leucocytes	4
	Scattered foci of macrophages and leucocytes are present	3
	One layer of macrophages and leucocytes are present	2
	Multiple layers of macrophages and leucocytes present	1
	Cannot be evaluated because of infection or other factors not necessarily related to the material	0

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 (μ CT) (SkyScan 1172 high-resolution micro-CT, SkyScan, Kontich, Belgium). High-resolution scanning was performed at energy of 100 kV and intensity of 98 μ A. 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 -2 θ diffractometer (XRD, Philips PW3710, Eindhoven, the Netherlands) using a $\text{CuK}\alpha$ -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

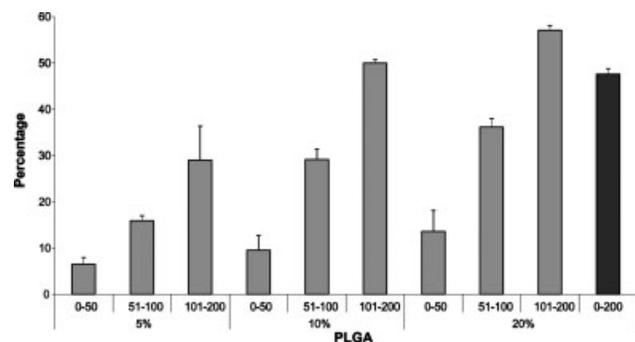


Figure 2. Microporosity (in %) of the different implant formulations. The 20% PLGA 0–200 μ m sizes were used for the early implantation periods (short-term biocompatibility) (CaP0/PGLA and CaP8/PLGA). All other formulations were used for 12- and 24-weeks implantation (long-term biocompatibility).

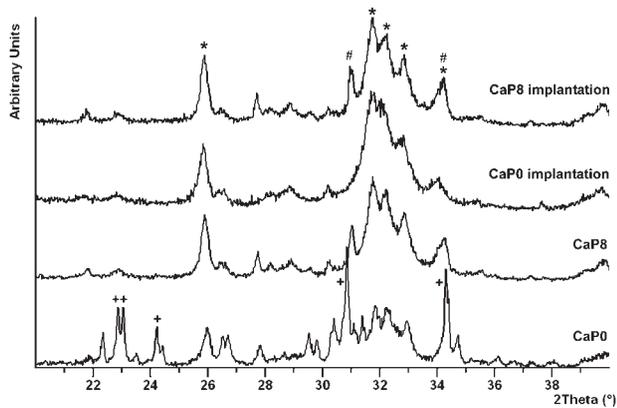


Figure 3. X-ray diffraction (XRD) patterns of the calcium phosphate cement discs. The XRD powder patterns of as-prepared (CaP0) and preincubated discs (CaP8), both before and after subcutaneous implantation for 12 days. (* = apatite; # = β -tri calcium phosphate (TCP); + = α -TCP).

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- μ m thick sections were prepared using a sawing microtome technique.¹⁷ 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).¹⁸ The presented data represent the average of these four measurements.

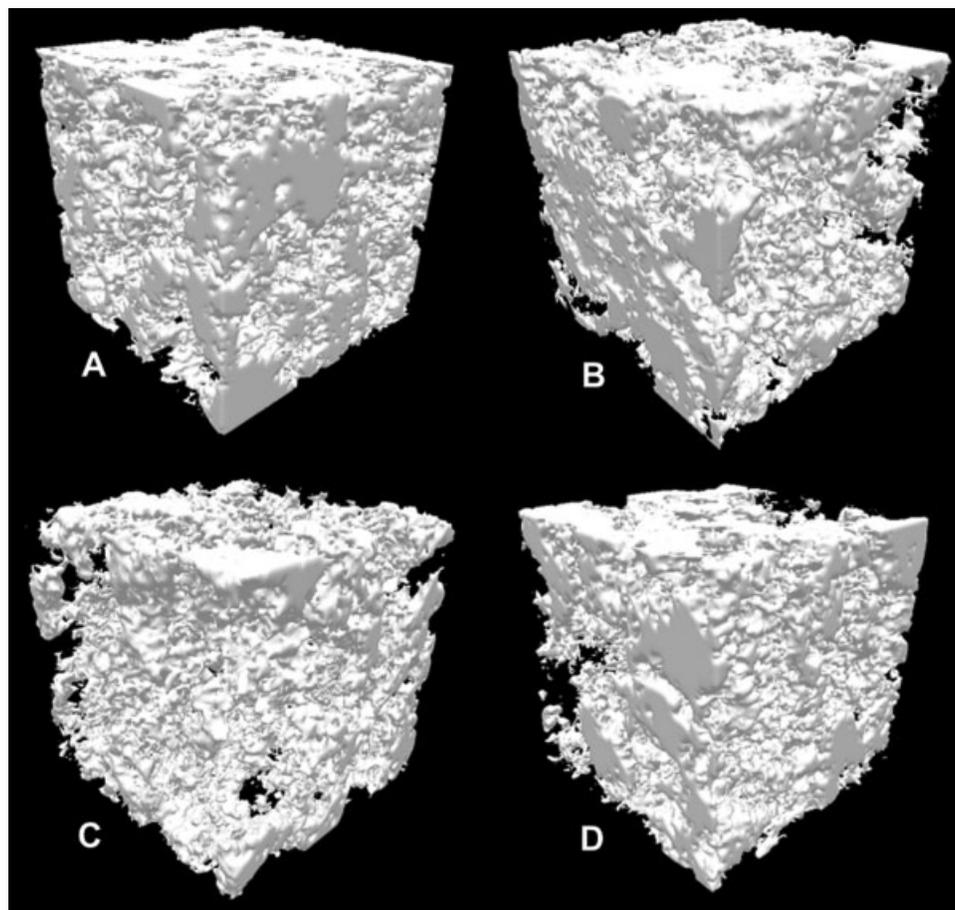


Figure 4. Microcomputed tomography (μ CT) images of CaP implants containing 5% (A), 10% (B), 20% (C) PLGA 101–200 μ m sizes. Together with 20% PLGA 0–200 μ m sizes (D) used for the early implantation periods (short-term biocompatibility). PLGA microparticles appear as pores in the CaP cement. The size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

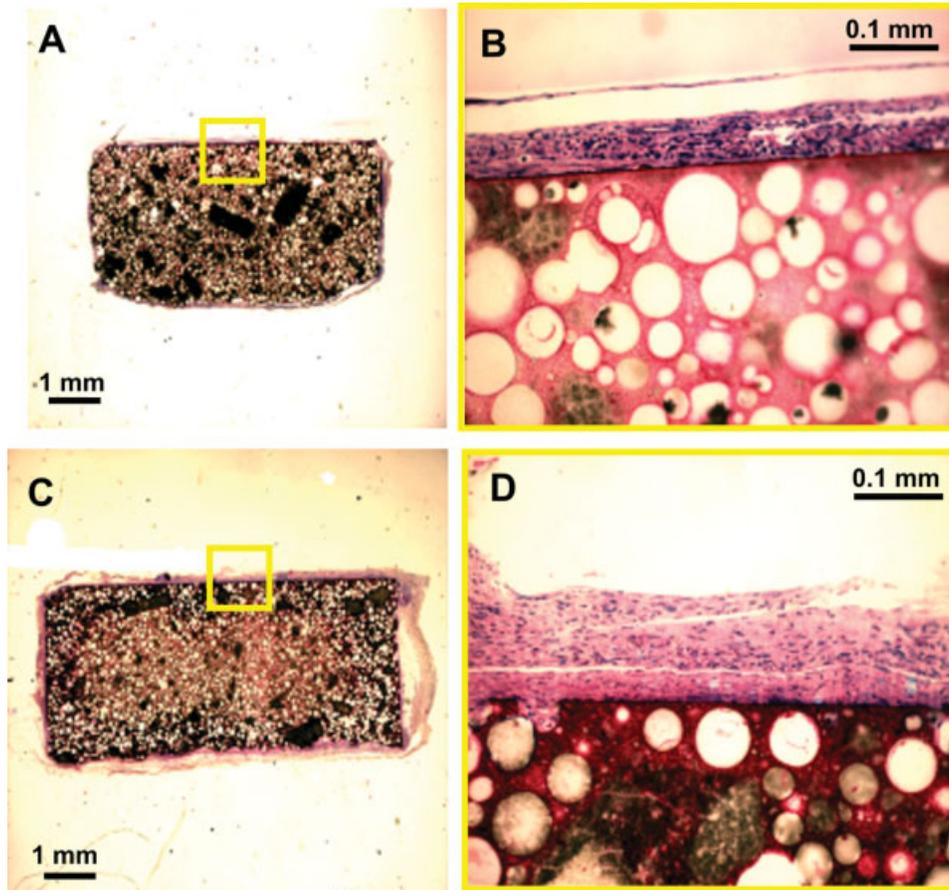


Figure 5. Histological sections of CaP implants containing 20% PLGA microparticles after 4 days (A and B) and 12 days (C and D) of implantation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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 α -tri calcium phosphate (α -TCP). Preincubation of the discs for eight weeks in water (CaP8) resulted in the hydrolysis of α -TCP into an apatite-like structure with some β -TCP. After implantation, the structure of the CaP8 samples remained the same, whereas the CaP0 samples underwent hydrolysis of α -TCP into an apatite-like structure without β -TCP

formation (Fig. 3). The incorporation of PLGA microparticles did not induce alterations of XRD patterns compared to pure CaP samples.

Microcomputed tomography (μ CT) indicated interconnectivity within all samples containing 20% (w/w) PLGA, except for those with small diameter (0–50 μ 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,

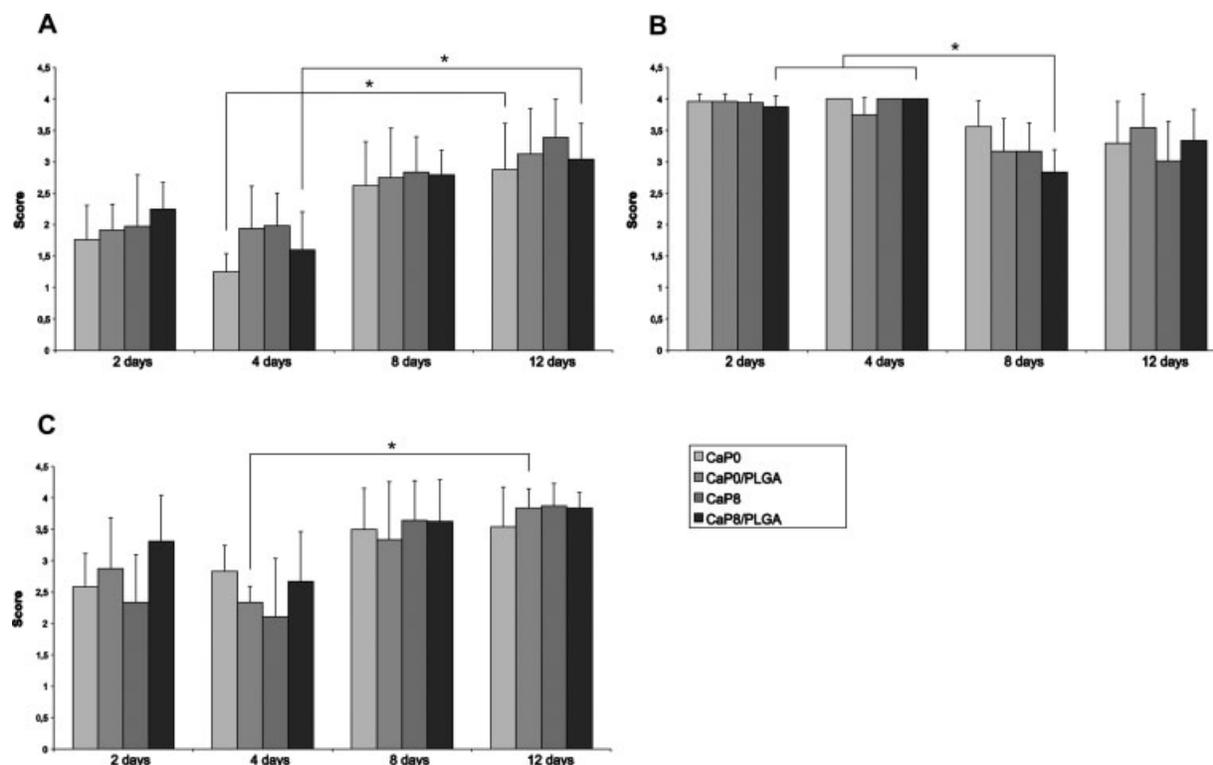


Figure 6. Histological evaluation of capsule quality (A), capsule quantity (B) and interface quality (C) of the short-term biocompatibility samples using the histological grading scale. Data shown represent mean \pm sd. Significant differences ($p < 0.05$) are indicated with an asterisk (*).

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,

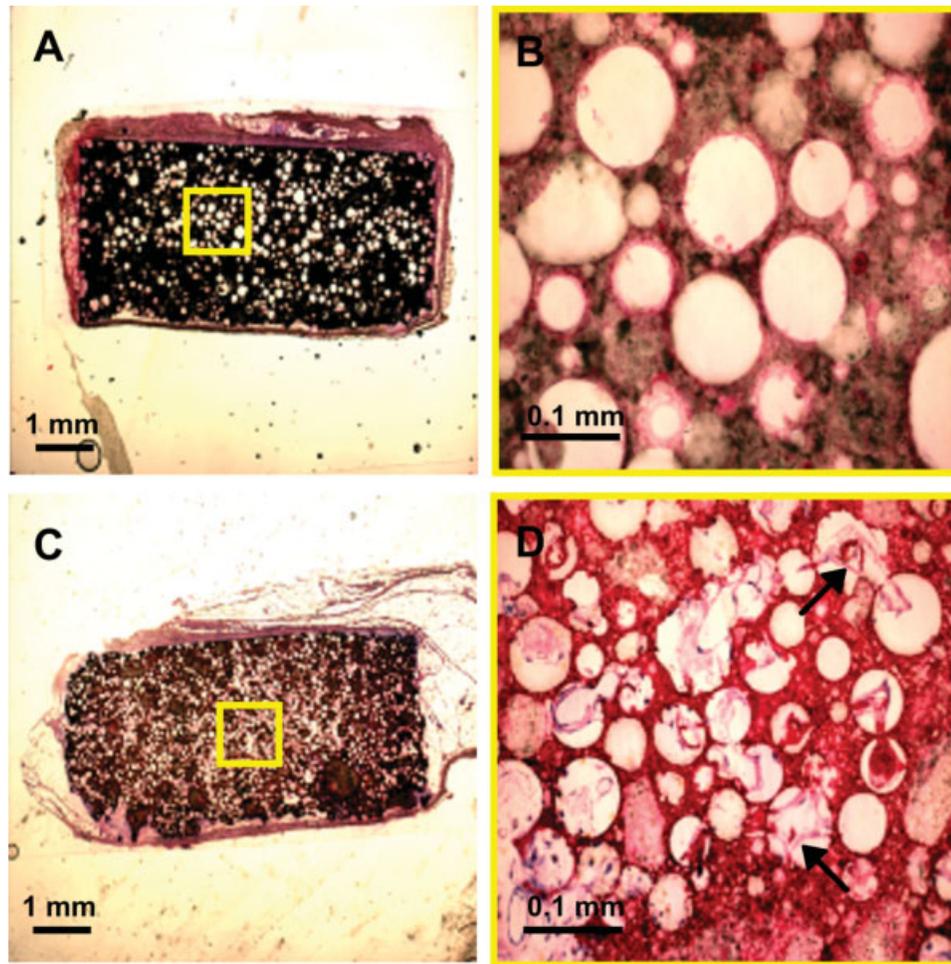


Figure 7. Histological sections of CaP implants containing 10% PLGA 101–200 μm sizes (A and B) and 20% PLGA 51–100 μm sizes (C and D) after 24 weeks of implantation. Arrows indicate fibrous tissue ingrowth. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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,¹³ which showed that without

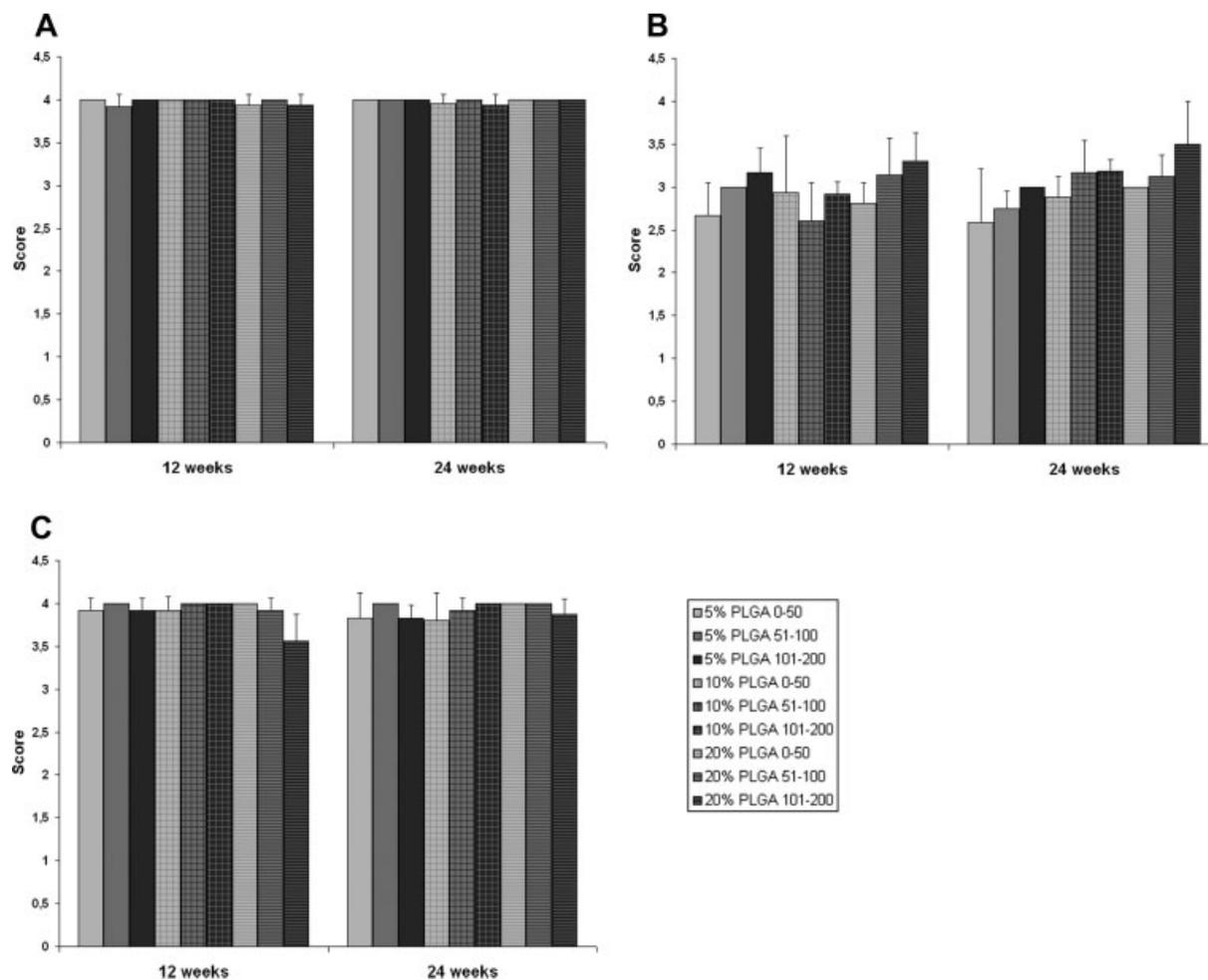


Figure 8. Histological evaluation of capsule quality (A), capsule quantity (B), and interface quality (C) of the long-term biocompatibility samples using the histological grading scale. Data shown represent mean \pm sd.

preincubation in water, CaP cement was not physicochemically 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 *in vitro*.¹³ Nevertheless, the *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 *in vivo* is higher than in *in vitro* experiments. Although PLGA is regarded as a nontoxic and biocompatible material,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.^{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 *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 *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.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 $50.1\% \pm 0.7\%$ in these implants was higher compared to the $36.2\% \pm 1.9\%$ 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 $50 \mu\text{m}$ to obtain optimal porosity of porous ceramics.²¹ Micro-computed 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 from 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.^{22,23} 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.¹⁰ However, that study consisted of smaller CaP/PLGA implants (2.0 mm height and 3.5 mm diameter) together with larger PLGA microparticles ($73 \pm 27 \mu\text{m}$) combined with CO_2 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.^{24,25}

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.

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