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Mechanical evaluation of implanted calcium phosphate cement incorporated with PLGA microparticles

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Abstract

In this study, the mechanical properties of an implanted calcium phosphate (CaP) cement incorporated with 20 wt% poly (DL-lactic-*co*-glycolic acid) (PLGA) microparticles were investigated in a rat cranial defect. After 2, 4 and 8 weeks of implantation, implants were evaluated mechanically (push-out test) and morphologically (Scanning Electron Microscopy (SEM) and histology). The results of the push-out test showed that after 2 weeks the shear strength of the implants was 0.44 ± 0.44 MPa (average $\pm$ sd), which increased to 1.34 ± 1.05 MPa at 4 weeks and finally resulted in 2.60 ± 2.78 MPa at 8 weeks. SEM examination showed a fracture plane at the bone–cement interface at 2 weeks, while the 4- and 8-week specimens created a fracture plane into the CaP/PLGA composites, indicating an increased strength of the bone–cement interface. Histological evaluation revealed that the two weeks implantation period resulted in minimal bone ingrowth, while at 4 weeks of implantation the peripheral PLGA microparticles were degraded and replaced by deposition of newly formed bone. Finally, after 8 weeks of implantation the degradation of the PLGA microparticles was almost completed, which was observed by the bone ingrowth throughout the CaP/PLGA composites.

On basis of our results, we conclude that the shear strength of the bone–cement interface increased over time due to bone ingrowth into the CaP/PLGA composites. Although the bone–cement contact could be optimized with an injectable CaP cement to enhance bone ingrowth, still the mechanical properties of the composites after 8 weeks of implantation are insufficient for load-bearing purposes.

Keywords: Injectable CaP/PLGA cement; Mechanical properties; Bone ingrowth

1. Introduction

Calcium Phosphate (CaP) ceramics are widely used as bone substitutes in dentistry, orthopedics and reconstructive surgery, because of their biocompatibility and osteoconductivity. Unfortunately, these ceramics are only available as prefabricated blocks or granules. Prefabricated blocks are difficult to shape, resulting in poor filling of the bone defect, while granules do not provide the dimensional stability and can easily migrate into the surrounding tissue. A solution for these problems can be CaP cement that can be shaped according to the defect dimension and harden in situ [1–9]. The injectable CaP cement as used in this study consists of a mixture of powder and liquid. The formed paste hardens in situ as a result of the entanglement of the newly formed crystals at body temperature. These cements are highly compatible with soft and hard tissues because of the apatitic nature of the setting reaction products. However, calcium phosphate ceramics are known as slowly biodegradable materials. Therefore, methods have been developed to enhance tissue ingrowth and degradation rate by increasing the porosity of the ceramics. Consequently, the creation of macroporosity in CaP cement will increase the degradation of the CaP cement as well as the ingrowth of new bone tissue into the cement porosity [10–12]. In view of this, cement composites were prepared in which poly (DL-lactic-*co*-glycolic) acid (PLGA) microparticles were incorporated [13–15]. The microparticles will be hydrolyzed in vivo and as a consequence create macroporosity. However, the in vivo degradation rate of the PLGA

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microparticles depends on the physicochemical properties such as molecular weight, particle size and structure [16]. Therefore, the inclusion of PLGA microparticles into CaP cement will result in increased macroporosity after degradation of the particles. The degradation rate of the microparticles is generally faster than the surrounding CaP cement. Additionally, the incorporation of degradable microparticles can be used to allow drug and growth factor delivery [15,17].

In vitro research on the mechanical properties of highly porous biodegradable cement composites by others showed compressive strength and a modulus of elasticity comparable to trabecular bone [18–29]. On the other hand, a previous study by Ruhé et al. [15] on the mechanical strength of CaP/PLGA cement composite scaffolds in vitro showed that the initial strength of this composite scaffold is significantly lower than for CaP cement alone. The creation of porosity is associated with a loss in mechanical strength of the cement material; this appears to make the material less suitable for use under loaded conditions. Alternatively, the initial decrease in mechanical strength could be compensated by the excellent bone biocompatibility of the material. This allows a fast ingrowth of bone into the cement porosity.

Therefore, the aim of our study is to prove that bone ingrowth results in an increase of mechanical strength of the macroporous cement.

2. Materials and methods

2.1. Substrates

CaP cement (Calcibon®, Merck biomaterial GmbH, Darmstadt, Germany) was used for the preparation of the implants. The chemical composition of this cement is 62.5% α-TCP, 26.8% $CaHPO_4$, 8.9% $CaCO_3$ and 1.8% PHA (α-TCP is α tri-calcium phosphate, PHA is precipitated hydroxyapatite). An aqueous solution of 1% Na_2HPO_4 was used as the liquid component. The ideal liquid/powder ratio for clinical applications has shown to be 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.

2.2. PLGA microparticles

PLGA (Purasorb®, Purac, Gorinchem, The Netherlands) 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 stirred 1000 ml beaker and another 394 ml of 0.3% PVA was added slowly. This was directly followed by adding 400 ml of a 2% isopropylic 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 suspension left 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. The microparticle sizes varied between 5 and 120 μm with an average size of 33 μm. This was determined with an optical microscope (Leica DM Microscope system, Microsystems AG, Wetzlar, Germany) after microparticles were suspended in deionized water. Digital image software (Leica Qwin®, Leica Microsystems AG, Wetzlar, Germany) was applied to determine the microparticle size distribution of the PLGA microparticles.

2.3. CaP/PLGA microparticle composites

CaP/PLGA cement composites were prepared by adding PLGA microparticles to the CaP cement powder in a weight ratio of 20–80%, respectively. Then, 350 μl cement liquid (1 wt% aqueous solution of Na_2HPO_4) was added to 1000 mg of the 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 mixing apparatus (Silamat, Ivoclar Vivadent AG, Schaan, Liechtenstein). After mixing for 15 s, the plunger was removed and the composite was injected in Teflon molds to ensure a standardized shape of the specimens. The disks (5 mm in diameter and 2 mm in height) were removed from the molds after setting of the cement at 37 °C for 1 h. The total porosity 67% was calculated by dividing the weight of CaP disks through the weight of CaP/PLGA disks, after the samples were placed in a furnace at 650 °C for 2 h to burn out the PLGA microparticles.

2.4. Surgery

Twenty-four male Wistar rats (250 g) were used for a cranial study. Each rat received one implant. The implantation periods were 2, 4, and 8 weeks, respectively ($n = 8$ for all implantation periods). National guidelines for the care and use of laboratory animals were respected. Surgery was performed under general inhalation anesthesia induced by 5% isoflurane, and maintained with 2.5% isoflurane by a non-rebreather mask. The rats were monitored with an oxy-pulse meter during surgery. To minimize postoperative pain, Fentanyl® (3 ml/kg intraperitoneal) was administered preoperatively and buprenorfine (Temgesic®) (0.05 mg/kg subcutaneous) for 2 days postoperatively.

After anesthesia, the rats were immobilized on their abdomen and the skull was shaved and disinfected with povidone–iodine. A longitudinal incision was made down to the periosteum from the nasal bone to the occipital protuberance, and soft tissues were sharp dissected to visualize the cranial periosteum. Subsequently, a midline incision was made in the periosteum, and the periosteum was undermined and lifted off the parietal skull. The pain was minimized by dripping lidocaine at the periosteum before incision. A full thickness bone defect was created in the parietal cranium, left of the sagittal suture, to avoid complications with the sagittal sinus. A hollow trephine bur (ACE Dental Implant Systems, Portugal) with an outer diameter of 5.1 mm in a dental handpiece was used to create the defect. The bone defect was carefully drilled under continuous cooling with physiologic saline and without damaging of the underlying dura. After that, the created bone segment was carefully removed. Following insertion of the implants, the periosteum was closed using non-resorbable Prolene® 5-0 suture material. Subsequently, skin was closed using resorbable Vicryl® 4-0 sutures.

The animals were housed individually in cages. The proper intake of fluids and food was monitored during the first 5 days post-operatively. Further, the animals were observed for signs of pain, infection and proper activity. After 2, 4 or 8 weeks of surgery rats were sacrificed by an overdose of CO_2.

2.5. Mechanical testing, i.e. push-out test

To determine the shear strength of the porous implants after 2, 4 and 8 weeks of implantation, a push out-test [34,35] was performed in a mechanical testing bench (MTS 858 Mini Bionix II, Gouda, The Netherlands). After sacrificing, the implants with their surrounding tissue

were retrieved and transported to the laboratory in PBS on ice. Subsequently, each specimen was fixated on a support jig with a hole 0.4 mm larger than the implant diameter (5.1 mm) to minimize the effect of the test condition on the push-out results. This support jig enabled the application of a vertical force (at a constant displacement speed of 0.5 mm/min) on the CaP/PLGA disks. When the peak force was reached (representing implant loosening), the test was immediately stopped to ensure minimal displacement of the disk. The shear strength of the bone–cement interface was calculated by dividing the push-out force (N) by π (pi) times the disc diameter (mm) times the cranial thickness (mm).

2.6. Scanning electron microscopy (SEM)

Following the push-out test, specimens were fixed in 10% formalin solution, dehydrated in a graded series of ethanol, and embedded in epoxy resin (Epofix®, Struers, Rødovre, Denmark). After polishing, the specimens were sputter-coated with gold, and examined with SEM (Jeol 6310 scanning electron microscope, Boston, MA, USA) to determine the fracture plane of the mechanically tested implants (e.g. in the cement, at the interface bone–cement, in the surrounding bone). SEM was performed at the Microscopic Imaging Center (MIC) of the Nijmegen Center for Molecular Life Sciences (NCMLS), The Netherlands.

2.7. Histology

After SEM examination, 10 μm-thick sections were prepared of the specimens embedded in epoxy resin in a direction transverse to the implant his axis using a sawing microtome technique. The sections were stained with methylene blue (cores) and basic fuchsin (collagen, bone), and investigated with a light microscope (Leica Microsystems AG, Wetzlar, Germany) to examine the bone–cement interface and possible bone ingrowth.

2.8. Statistical analyses

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

3. Results

3.1. Push-out test

The results of the push-out test (Fig. 1) demonstrated that after 2 weeks of implantation the push-out values ranged from 0.18 to 1.28 MPa, which increased from 0.37 to 3.12 MPa at 4 weeks and finally got to a range varying from 0.21 to 8.12 MPa. Calculating these push-out results into the shear strength of the bone–cement interface resulted in averages ($\pm$standard deviation) of 0.44 ± 0.44 MPa at 2 weeks, which increased to 1.34 ± 1.05 MPa at 4 weeks and finally resulted in 2.60 ± 2.78 MPa at 8 weeks. However, the differences between the three implantation groups were not significant.

3.2. SEM examination

SEM examination (Fig. 2(A)) showed a fracture plane at the bone–cement interface at 2 weeks. The 4-week implants showed a fracture plane, which was found at the bone–cement interface and into the CaP/PLGA composites as well (Fig. 2(B)). The 8-week implants demonstrated only fractures throughout the CaP/PLGA composites (Fig. 2(C)).

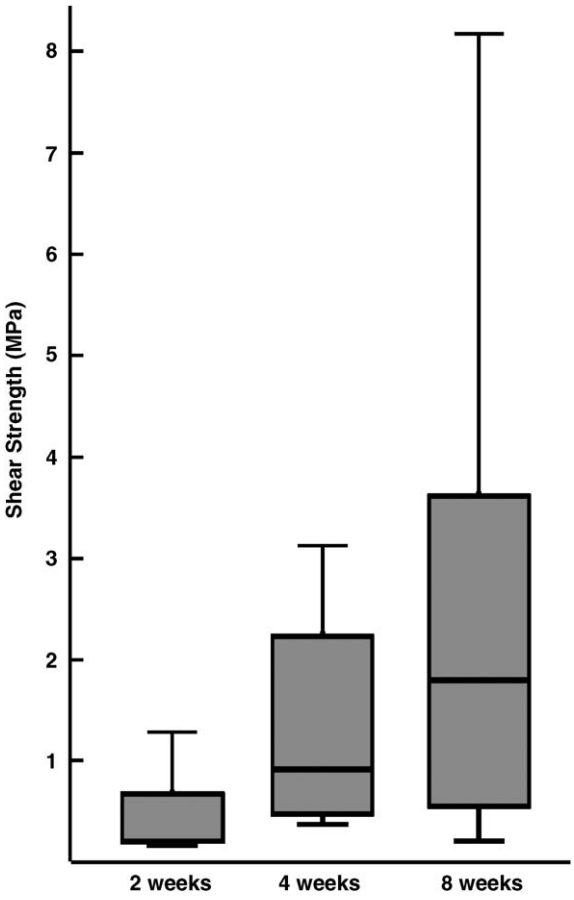

Fig. 1. Box–Whisker plot of the push-out results from the 2, 4 and 8 week implantation periods. The box shows the first and third quartile with the median value in between and the whiskers are representing the minimum and maximum values.

3.3. Histology

Histological evaluation (Fig. 3(A and B)) showed that the 2 weeks implantation period resulted in minimal bone ingrowth from the cranial bone into the implants. At four weeks of implantation, the peripheral PLGA microparticles were degraded and replaced by newly formed bone. Further, a small layer of bone was present at the cerebral side of the implants. Fibrous tissue was observed between the cranial bone and the CaP/PLGA composites, especially when no close contact of cranial bone with the implant was observed. After 8 weeks of implantation, almost all PLGA microparticles were degraded and bone deposition was observed throughout the composites. The CaP cement was starting to degrade as indicated by loss of integrity of the implant and by the replacement of the cement with newly formed bone (Fig. 3(C and D)). Nonetheless, not all of the implants showed good bone ingrowth, some implants showed fibrous tissue between the cranial bone with the

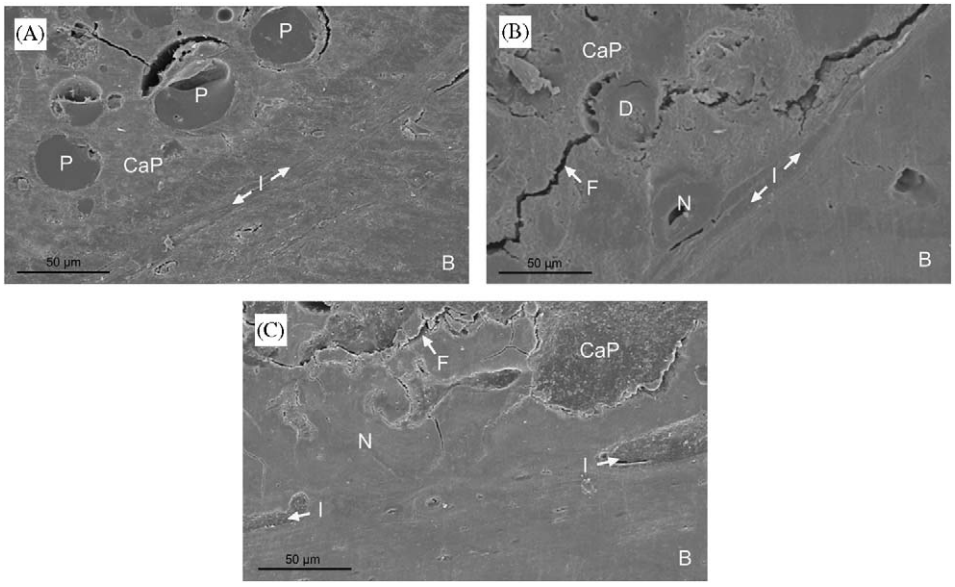

Fig. 2. SEM examination of a 2 (A), 4 (B) and 8 (C) week implantation period, respectively. CaP = calcium phosphate cement; B = cranial bone; D = degradated PLGA microparticle; F = fracture plane; I = bone–cement interface; N = newly formed bone; P = PLGA microparticle.

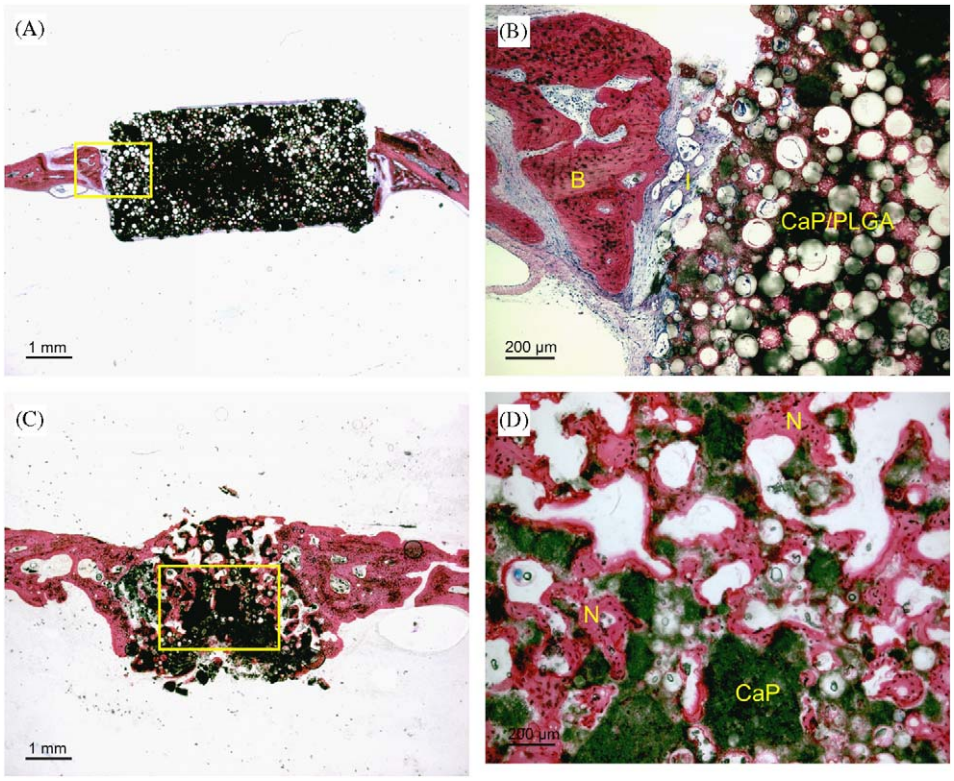

Fig. 3. Histology of a two (A and B) and 8 (C and D) week implantation period, respectively. B = cranial bone; I = bone–cement interface (filled with fibrous tissue); N = newly formed bone.

CaP/PLGA composites when no close contact between bone and implant was observed.

4. Discussion

FDA regulations concerning medical devices state that bone fillers should be investigated on their mechanical properties to gain approval for clinical usage. The biomechanical testing should demonstrate the quality of the newly formed bone (Class II Special Controls Guidance Document: Dental bone-grafting material devices). Therefore a push-out test was used to evaluate the shear strength of the bone–cement interface. Results of the push-out test showed that shear strength of the implants increased

during the 8 weeks implantation period. The 8-week specimens with the highest shear strength still maintained the bone–cement bond, although the composites were fractured. This indicated that the 8-week implantation results were an underestimation of the real shear strength value. Nevertheless, these results confirmed our hypothesis that degradation of PLGA microparticles resulted in macroporosity of the CaP cement, followed by replacement of newly formed bone, and as a consequence in an increase of mechanical properties. This effect was also described in other studies involving CaP cement [30,31].

To avoid complications with the sagittal sinus, the cranial defect size used in this study was not a critical size defect [32,33], but, this was not a restriction for the aim of the study, i.e. the mechanical properties of CaP cement incorporated with PLGA microparticles. Also, pre-set CaP/PLGA implants were used in this study, because the rat cranial defect is not suitable to inject composites. Furthermore, problems with the final setting time were still present in vivo, which could result in non-standardized implants.

In this study, results from the push-out test assumed an equal load distribution at the bone–cement interface. However, test boundary conditions can significantly influence the load distribution [34]. Nevertheless, these push-out results are still an approximation of the real bone–cement interface shear strength.

Interestingly, large shear strength variations between samples from the same implantation period were observed. This variation can be explained by the differences in random distribution of the PLGA microparticles in the CaP cement. Accumulation of PLGA microparticles can result in relatively weak spots inside the composite, resulting in reduced push-out strength values. Large variations of the push-out test can also be due to differences between rats, surgical techniques, surface roughness or implant locations [35]. Some implants were press-fitted into the cranial defect, where others were less press-fitted. More bone formation was observed with the histological sections when close contact between implant and bone was detected, compared to implants with less contact between the implant and the cranial bone. Dhert et al. reported that mechanical test results can be influenced by implant fixation [34]. In the future, this problem can be solved by applying CaP/PLGA composites as an injectable composite and not as pre-set disks.

For this study, CaP/PLGA composites with weight ratios of 80–20%, respectively were used. This composition was chosen based on previous research with similar CaP/PLGA cement composites. Previous in vitro research showed that PLGA microparticles started to degrade from week 6 and degradation was completed at week 12. Degradation of the PLGA microparticles resulted in reduction of the mechanical properties of the implants. This was also indicated in in vitro compression tests, in which the composites had an initial 30 MPa strength which was reduced to 4.3 MPa at 12 weeks [36]. Therefore, based on the in vitro compression tests and the results of the push-out test performed in the present study, we concluded that the CaP/PLGA composites are not suitable for load bearing purposes, since it was reported that the shear strength of compact bone ranges from 53.1 to 70 MPa [37]. Lowering the PLGA ratio in the composites could be considered to increase the mechanical properties of the implants, but this reduces the amount of bone ingrowth in the composites.

Other factors, which influence the mechanical properties of the composites, are the total amount of porosity, pore size and pore structure [38]. These factors should be appropriate to allow cell ingrowth and transport of nutrients and waste [39]. In this study, the total porosity of the CaP/PLGA composites was approximately 67%, with microparticle sizes varying from 5 to 120 μm with an average diameter of 33 μm. These microparticle sizes were a result of the forcefulness of vortexing during the double emulsion process, which was crucial for the eventual size distribution. The size distribution of the microparticles was measured afterwards. This could also explain why different microparticle sizes, varying from 0.017 mm to 0.1 mm, were used in previous studies [10,14,15] and this study. However, PLGA microparticles were added based on their weight, not on their size, so the amount of PLGA in the implants remained the same, independent of the microparticle size. Nevertheless, the microparticles in our study create a less than optimal pore size for bone ingrowth as described by literature. Research by others on the optimal pore size of porous ceramics varied between 50 and 400 μm [38,40,41]. Although, the average diameter is much smaller than the reported optimal size, there is still abundant ingrowth of bone in our specimens. This suggests that the optimal pore sizes may be much smaller for CaP cement than for materials with less favorable biological interactions.

Further, porosity greatly influences the rate of degradation of the CaP cement. It was reported that degradation of macroporous CaP cement can reach up to 80% after 10 weeks [10]. However, crystallinity, density, pH in the implant region, animal model and implantation site also contribute to the rate of degradation [40]. Therefore, complete degradation of the CaP cement varies between weeks to years depending on the physicochemical properties being used [42–45].

The increasing shear strength of the bone–cement interface over time was also confirmed by SEM examination, which showed a fracture plane at the bone–cement interface at 2 weeks, while the 4- and 8-week specimens created a fracture plane into the CaP/PLGA composites, indicating an increased strength of the bone–cement interface. Histological evaluation confirmed that the 2 weeks implantation period resulted in minimal bone ingrowth. At 4 weeks of implantation, the peripheral PLGA microparticles were degraded and replaced by newly formed bone. Further, a small layer of bone was present at the cerebral side of the implants bridging the entire defect. Fibrous tissue was observed between the cranial bone and

the CaP/PLGA composites, especially when no close contact was observed. After 8 weeks of implantation, almost all PLGA microparticles were degraded and bone deposition was observed throughout the composites. SEM and histology revealed that bone formation started at the defect edges and cerebral side of the implant and proceeded into the macropores which were created by the degradated PLGA microparticles. This observation agrees with previous in vivo experiments [10,14] which showed that this CaP cement showed excellent bone compatibility, but full comparison with both studies is not possible, since different composite formulations, different implants sizes and longer implantation periods were used.

5. Conclusions

On basis of our results, we conclude that the shear strength of the bone–cement interface increased over time due to bone ingrowth into the CaP/PLGA composites. Although the bone–cement contact could be optimized with an injectable CaP cement to enhance bone ingrowth, the mechanical properties of the composites after 8 weeks of implantation are still insufficient for load-bearing purposes.

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