

# Creation of macroporous calcium phosphate cements as bone substitutes by using genipin-crosslinked gelatin microspheres

Meng Li · Xingyan Liu · Xudong Liu ·  
Baofeng Ge · Keming Chen

Received: 2 July 2008 / Accepted: 17 November 2008 / Published online: 4 December 2008  
© Springer Science+Business Media, LLC 2008

**Abstract** Macroporous calcium phosphate cements (CPCs) were developed using genipin-crosslinked gelatin microspheres (GMs) with two weight ratios (2.5 wt% and 5 wt%). The initial setting time of the composite was prolonged by GMs. After GMs/CPCs were soaked in phosphate-buffered saline (PBS) for several weeks, macropores appeared as a result of the degradation of GMs. The presence of GMs accelerated the setting reaction and improved the structure of the composite. The compressive strength increased up to 12 MPa (2.5 wt% GMs/CPCs) and 14 MPa (5 wt% GMs/CPCs) after one week of PBS soaking, then gradually decreased to 9 MPa (2.5 wt% GMs/CPCs) and 7 MPa (5 wt% GMs/CPCs) after three weeks of soaking, and further to 6 MPa (2.5 wt% GMs/CPCs) and 2 MPa (5 wt% GMs/CPCs) after five weeks of soaking. CPCs with 2.5 wt% GMs were the most favorable composite in the tested samples. Cell experiments showed that rat osteoblasts displayed normal morphologies when exposed to the 2.5 wt% GMs/CPCs, and proliferation of the cells was also enhanced. An *in vivo* study showed that new bone tissue was able to grow into the pores that resulted from GM degradation. This study suggests that the new composite could be a promising candidate for use as a bone substitute under non-compression-loaded circumstances.

## 1 Introduction

Calcium phosphate cements (CPCs) can be considered as good candidates for bone substitutes with several advantages; for example, they are injectable, osteoconductive, and easy to shape [1, 2]. However, their slow resorption *in vivo* prevents them from being widely used in orthopedics or to treat other conditions because the growth process of new bone tissue may be delayed due to the presence of the CPCs [2, 3].

The resorption of CPCs can be passive or active. Passive resorption is due to dissolution of the material in body fluids [4]. Active resorption is the primary process and is due to cellular activities [5, 6]. Small particles can be phagocytized by multinucleated cells such as macrophages and foreign body giant cells, and larger volumes of material are resorbed by osteoclasts. This kind of resorption usually only occurs at the bone-cement interface [5, 6]. Since CPCs do not have a macroporous structure, the bone-cement surface area is relatively small, making the resorption rate of the cements quite low [2, 7, 8]. Meanwhile, it has been confirmed that highly macroporous structures in the improved CPCs accelerated the rate of the resorption *in vivo* [9, 10].

Several methods have been developed to improve the resorption of the CPCs by introducing macropores into the materials. Mixing the CPCs with highly water-soluble sucrose or mannitol crystals has been a popular method [11–14], and a CO<sub>2</sub>-gas bubble method or air bubble trapping was also widely used [10, 15, 16]. However, the composites produced by these methods had poor mechanical properties in the early period when the ingrowth of bone tissues did not occur [10–16]. Recent studies have focused on introducing macroporosity into CPCs by using absorbable microspheres, such as PLGA

---

M. Li  
Orthopedic Institute of Chinese People's Liberation Army,  
Xijing Hospital, Fourth Military Medical University,  
Xi'an 710032, People's Republic of China

X. Liu (✉) · X. Liu · B. Ge · K. Chen  
Orthopedic Trauma Institute of Chinese People's Liberation  
Army, Lanzhou General Hospital, Lanzhou,  
Gansu Province 730050, People's Republic of China  
e-mail: Xingyanliu@2008.sina.com

(poly(D,L-lactic-co-glycolic) acid) microspheres [9, 17–21]. Degradation of the microspheres would not only create macropores, but the inclusion of bone morphogenetic protein 2 (BMP-2)-loaded microspheres within CPCs may also be a promising method to augment the osteoinduction of the composite because these microspheres showed good controlled release of BMP-2 [18–21]. Though the initial mechanical properties of the CPCs with microspheres were stronger than previous macroporous CPCs, the results are still not satisfying [9, 17, 18, 20].

Gelatin is created from the physical or chemical degradation of collagen, which is the main organic component of bone tissues. Previous studies [22–25] have demonstrated that the microstructure of calcium phosphate cement could be strengthened by incorporating gelatin (A- or B-type degradation of collagen type I from porcine or bovine skin) into the cement. The activity and differentiation of osteoblasts were also improved by gelatin-enriched cement [26, 27]. On the other hand, gelatin microspheres (GMs), which have been widely used as a controlled release carrier of growth factors, showed good biocompatibility and degradability *in vivo* when they were crosslinked by genipin [28, 29].

In the present study, porous CPCs were formulated by mixing genipin-crosslinked GMs with cement powders, and a series of tests were then conducted to investigate the physical properties and biocompatibility of GMs/CPCs. We hypothesized that inclusion of GMs into CPCs would result in enhanced initial mechanical properties and biocompatibility, and that macroporosity of the composite would also be improved after GM degradation.

## 2 Materials and methods

### 2.1 Preparation of genipin-crosslinked GMs

GMs were prepared by using an emulsification–solvent extraction method, which has been reported in the literature with some modifications [28–30]. Briefly, type B gelatin (1.5 g, degradation of collagen type I, from bovine skin, 225 Bloom, Sigma-Aldrich Corporation, St. Louis, MO) was dissolved in 10 ml phosphate-buffered saline (PBS, pH 7.4) in a water bath at 50°C. The gelatin solution was added into 50 ml corn oil, which was preheated to 50°C. The biphasic system (corn oil and aqueous solution containing gelatin) was thoroughly mixed to form a water/oil emulsion using a propeller. Subsequently, the emulsion system was chilled to 4°C in a refrigerator, and GMs were formed in the aqueous phase. The obtained GMs were then rinsed in acetone several times to remove the remaining oil on their surfaces. Finally, the rinsed GMs were vacuum dried overnight.

To crosslink GMs, the samples were dispersed into 5 wt% genipin (Wako, Japan) in an aqueous ethanol solution (70% ethanol by volume) for about 72 h at 37°C. Crosslinked GMs were rinsed for 4 h with an aqueous ethanol solution (99.5% ethanol by volume) to remove the residual crosslinking agent on their surfaces. Subsequently, the rinsed GMs were vacuum dried for 24 h to evaporate ethanol. The degree of crosslinking was determined by ninhydrin assay [28]. Previous studies [28, 29] showed that GMs with a 50% degree of crosslinking could be totally degraded after four weeks in the body environment, but GMs with a 60% degree of crosslinking could maintain good sphericity for 21 days. Therefore, we selected the GMs with a 60% degree of crosslinking, assuming that they would be gradually degraded over more than 4–5 weeks in the body environment.

The crosslinked and uncrosslinked GMs were sprinkled onto a double-sided adhesive tape fixed to an aluminum stage and spattered with gold film. The morphologies of microspheres were examined with a scanning electron microscope (SEM, Model JSM-5600 and JSM-6701, Jeol, Japan).

### 2.2 Preparation of GM/CPC composites

CPC powders, consisting of an equimolar mixture of tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA) (Shanghai Rebone Biomaterials, China), were mixed with genipin-crosslinked GMs by using a conditioning mixer (ARE-250, Thinky, Tokyo, Japan). Eventually, GM/CPC ratios of 2.5 wt% and 5 wt% were obtained. Cement without GMs was used as a control, and the setting liquid was 1 M Na<sub>2</sub>HPO<sub>4</sub> (Shanghai Rebone Biomaterials, China). The cement specimens were prepared by using a liquid-to-powder (L/P) ratio of 0.4 ml/g. After mixing, the specimens were placed into cylindrical stainless steel molds to form specimens with dimensions of 5 mm in diameter and 10 mm in height, which were then stored in an incubator at 100% relative humidity and 37°C for 2 h. The samples were then demolded and soaked in 5 ml of PBS (pH: 7.4) and incubated at 37°C in a water bath on a shaker table (70 rpm) for one, three, or five weeks [18, 31]. The sample buffer was refreshed every three days.

### 2.3 Physical properties of GM/CPC composites

The setting time of the various cement formulations was assessed by using a custom Gillmore method. Six parallel experiments were carried out for every group. After different soaking times (one week, three weeks, and five weeks) at 37°C, the setting specimens were removed from the PBS and the compressive strength of the wet samples

was measured using a universal testing machine (AG-2000G, Shimadzu Autograph, Shimadzu Corporation) at a loading rate of 0.5 mm/min. Then, the morphology of the fracture surface was observed with a scanning electron microscope (SEM, Model JSM-5600 and JSM-6107, Jeol, Tokyo, Japan) after drying at 120°C. For the purpose of X-ray diffraction analysis, the fracture specimens were immediately immersed in liquid nitrogen for 10 min in order to end the setting reaction. All composites were ground into powders for characterization using an X-ray diffractometer (XRD, Shimadzu XD-D1, Kyoto, Japan) operated at 40 kV and 60 mA at a scanning speed of 2°/min in order to determine phase composition of the products.

In the present experiment, macroporosity can be regarded as the porosity of the cement formulations in which the pores are created by the erosion of the GMs. Therefore, the GM/CPC samples were weighed after one week, three weeks, and five weeks of soaking, and their volumes were precisely measured. Equations 1–3 were derived for calculation of the total porosity and the macroporosity.

$$P_{\text{TOTAL}} = (d_{\text{HA}} - d_{\text{MEASURED}})/d_{\text{HA}} \quad (1)$$

$$P_{\text{MACRO}} = 1 - d_{\text{MEASURED}}/d_{\text{CPC}} \quad (2)$$

$$d = W/V \quad (3)$$

where  $P_{\text{TOTAL}}$  is the total porosity (%),  $P_{\text{MACRO}}$  the macroporosity (%),  $d_{\text{HA}}$  the density of hydroxyapatite (3.14 g/cm<sup>3</sup>),  $d_{\text{MEASURED}}$  the density of the measured sample,  $d_{\text{CPC}}$  the density of the CPC sample,  $W$  the weight of the sample, and  $V$  the volume of the sample.

#### 2.4 Biocompatibility assays

In this study, CPCs with 2.5 wt% GMs showed good general physical properties. Thus, this composite was used in the following experiment. CPCs served as the negative control, and an empty well of tissue culture polystyrene (TCPS) as the biocompatible control. The cement specimens for the cell attachment study were bar-shaped with dimensions of 3 mm × 4 mm × 12 mm and were sterilized by autoclaving at 121°C for 20 min [32]. Some of these specimens were immersed in a well with 4 ml of fresh medium (without cells) and extracted overnight in the incubator to accumulate any possible harmful leach-out in the medium [32]. Then the extraction media was collected and used for cell experiments. Rat osteoblasts were isolated from the calvaria of neonatal Sprague-Dawley rats by digestion in collagenase solution for 2 h. The cells from the digestion were pooled, washed, and resuspended in tissue culture medium (Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 1% antibiotics), and then cultured in a humidified 5% CO<sub>2</sub> balance air

incubator at 37°C [33]. The second passage of the cells was used for the experiment. Cell suspension was adjusted to a density of 1 × 10<sup>4</sup> cells ml<sup>-1</sup> and 100 μl of the cell suspension was added to each well of a 96-well plate (Nunc, Denmark). After 24 h of culturing, the medium was replaced by 100 μl extraction fluid of the composite. After incubation for one, three, or five days, the extraction fluid was removed and 100 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (MTT, Sigma, 0.5 mg ml<sup>-1</sup> in PBS) was added. Four hours after the solution was added, the medium was removed and purple formazan crystals were solubilized with 100 μl dimethyl sulfoxide (Amresco) at room temperature. The optical density (OD) was read at 590 nm wavelength in a microplate reader (ELX 800, Bio-TEK, USA). Six samples per group were tested in the experiment. In addition, cells were plated in the wells of a 24-well plate that was coated with the 2.5 wt% GMs/CPCs or the CPC control. Three days after plating, the cells were rinsed with PBS for 10 min, fixed with 3% glutaraldehyde in 0.01 M PBS for one hour, and were subjected to graded alcohol dehydrations (30%, 50%, 70%, 90%, 95%, and 100%, each for 10 min). Scanning electron microscopy was used to observe the morphology of cells grown on the surfaces of the composites after fixed samples were coated with gold.

In the animal study, eight mature goats were randomly assigned to two treatment groups and received cylinders of CPCs or 2.5 wt% GMs/CPCs. Two nonadjacent lumbar vertebral bodies were treated with the assigned materials. Animals were anesthetized, and placed in the right lateral recumbency. The vertebral body was exposed through a lateral retroperitoneal approach. A piece of cortical bone in the lateral vertebral was excised, and a trabecular bone defect was created with dimensions 5 mm in diameter and 10 mm in length. After it was irrigated and dried, the defect was plugged with the materials, and tissues were closed in layers. Six weeks after the operation, the treated vertebral bodies were harvested after euthanasia. 14 days or 4 days before animals were killed, calcein (5 mg/kg) or tetracycline (20 mg/kg) was injected into the subcutaneous tissue of the animals. Half of the vertebral bodies were bisected along the longitudinal axis, fixed in 70% ethyl alcohol, and dehydrated in graded solutions of ethyl alcohol (70%, 95%, and 100%), and then embedded in methyl methacrylate. Sections of 30 mm thickness were cut using a bone saw (Leica SP1600, German) and observed by fluorescence microscope immediately thereafter. After staining with a modified ponceau trichrome bone stain, sections were observed under light microscope. The rest of the samples were fixed in formaldehyde and embedded in paraffin after decalcification and dehydration. Then, 5 mm sections were created and observed after HE stain.

## 2.5 Statistical analysis

Statistical analysis was performed with a computerized statistical program (Statistical Analysis System, Version 6.08, SAS Institute Incorporation, Cary, NC) using one-way analysis of variance (ANOVA). Tukey's multiple comparison test was used for post hoc analysis, with significance assumed at  $P < 0.05$ .

## 3 Results

### 3.1 Preparation of genipin-crosslinked GMs

SEM micrographs of the prepared GMs before and after crosslinking are shown in Fig. 1. The prepared GMs were spherical in shape and had an average diameter of  $45 \pm 25 \mu\text{m}$ . Genipin-crosslinked GMs did not induce a significant change in their morphologies, but the microspheres before crosslinking were smoother than after crosslinking.

### 3.2 Physical properties of CPCs and GM/CPC composites

Initial setting time of the control cement was within 8 min ( $7.1 \pm 0.8$  min). In contrast, both of the GMs/CPCs took a remarkably long time of  $10.2 \pm 0.9$  min (2.5 wt%) and

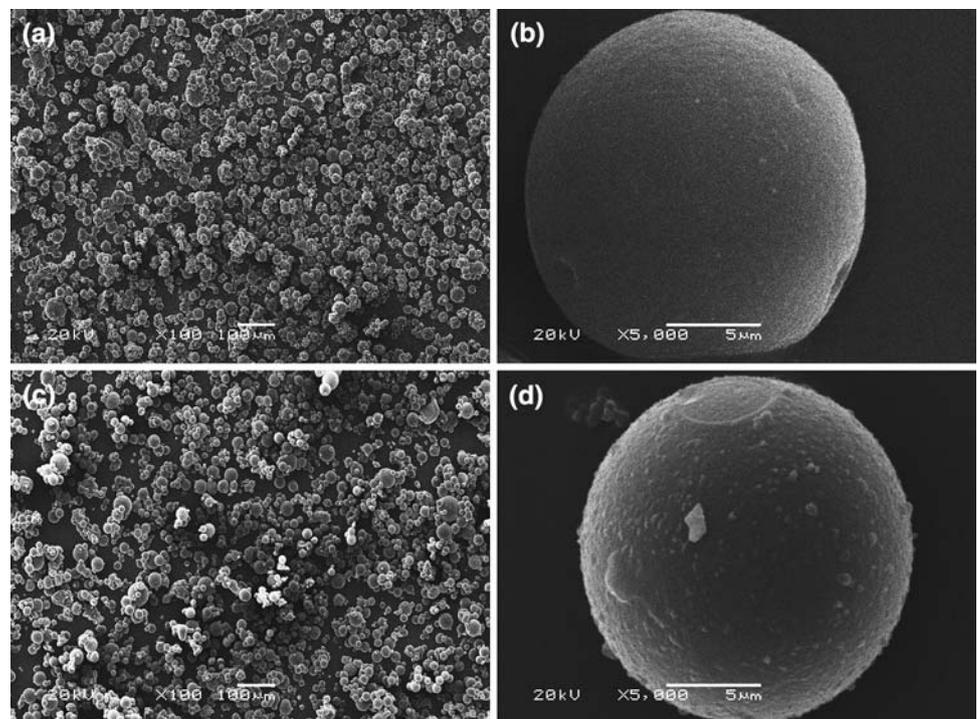
$13.3 \pm 1.4$  min (5 wt%) to harden. These values are significantly different from each other ( $P < 0.05$ ).

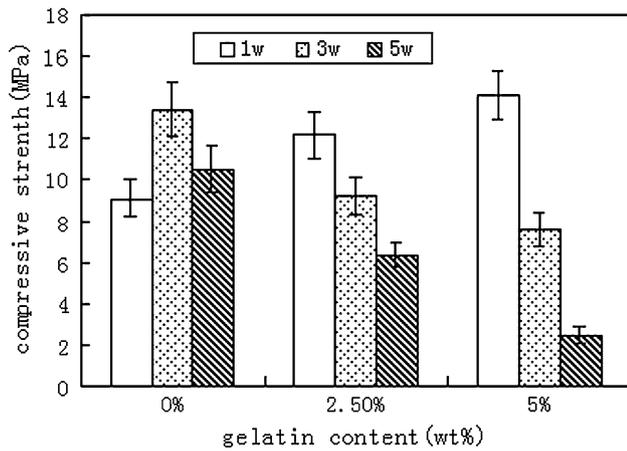
Compressive strengths of three composites are plotted in Fig. 2. After one week of soaking, GMs/CPCs had a strength of  $12.2 \pm 1.1$  MPa (2.5 wt%) and  $14.1 \pm 1.2$  MPa (5 wt%), values that were significantly higher than the  $9.1 \pm 0.9$  MPa of the CPCs ( $P < 0.05$ ). When the GMs were gradually degraded by immersion, the compressive strength of GMs/CPCs slowly decreased. After three weeks of soaking, the compressive strength of GMs/CPCs decreased to  $9.2 \pm 0.9$  MPa (2.5 wt%) and  $7.6 \pm 0.8$  MPa (5 wt%), while after five weeks, they were  $6.3 \pm 0.6$  MPa for 2.5 wt% GMs/CPCs and  $2.5 \pm 0.4$  MPa for 5 wt% GMs/CPCs, values that were significantly lower than that of the CPCs ( $P < 0.05$ ).

In Table 1, the porosity and macroporosity of the samples are given. The CPCs had a porosity of about 40%, and did not have a macroporosity. With the degradation of GMs, a higher porosity was obtained in both GMs/CPCs, and a macroporosity was also observed. In all three time points, the porosity and macroporosity of the GMs/CPCs were higher than that of the CPCs, and the 5 wt% GMs/CPCs had the highest porosity and macroporosity.

Micrographs of the composites are showed in Fig. 3. After soaking in PBS, a macroporous structure took form on the surfaces of both GM/CPC samples and macroporosity increased with the soaking time. After one week of soaking, many GMs could be seen on the surfaces of the

**Fig. 1** SEM micrographs of gelatin microspheres prepared in the study before crosslinking (a, b) and after crosslinking (c, d)





**Fig. 2** Compressive strength of CPCs and GMs/CPCs after weeks of soaking

**Table 1** Porosity and macroporosity of the composites for weeks of immersion

Tested Group	Porosity (macroporosity) after immersion		
	1 week	3 weeks	5 weeks
CPCs control	40.2 ± 0.8 (-)	38.9 ± 0.7 (-)	40.5 ± 0.74 (-)
2.5 wt% GMs\CPCs	44.6 ± 0.72 (9.6 ± 0.12)	56.3 ± 0.46 (22.8 ± 0.37)	65.5 ± 1.12 (39.8 ± 0.47)
5 wt% GMs\CPCs	47.8 ± 0.62 (12.7 ± 0.13)	61.3 ± 0.54 (29.6 ± 0.33)	70.2 ± 1.21 (44.6 ± 0.42)

GM/CPC samples, and very little erosion was observed (Fig. 3a, b). Three weeks later, bulk erosion was visible, which resulted in many macropores, and the remaining microspheres had lost their sphericity (Fig. 3c, d). After five weeks, the particles were almost completely eroded and a structure of interconnective macropores was observed in which some traces of GMs could be found (Fig. 3e, f). However, there were no macropores observed on the surface of the CPCs after five weeks of soaking (Fig. 3g). In the high power photos, after one week, more dense structures and more regular crystals could be seen in the GM/CPC samples (Fig. 3h–j), particularly in the macropores formed after GM erosion.

X-ray diffraction analysis was carried out on the cements after incubation in PBS for different periods. This analysis indicated that the presence of gelatin accelerated the transformation of cements into apatites. The powder X-ray diffraction patterns showed that the relative peak intensities of the TTCP in the CPCs were higher than that of the GMs/CPCs, and all of the specimens showed the peak of hydroxyapatite after one week of soaking (Fig. 4a–c). It took five weeks for 2.5 wt% and 5 wt% GMs/CPCs to form complete apatite as the end-products (Fig. 4c), while for the control CPCs, there was still a small TTCP content.

### 3.3 Biocompatibility assays

Cell biocompatibility assays, which were performed at different time periods, are displayed in Table 2. The cell proliferation in the presence of the GMs/CPCs was significantly higher than that of the CPCs at the same time point. However, we observed no differences between GMs/CPCs and TCPS. After three days of culture, SEM examination showed normal and polygonal morphology of the cells on both composites. In agreement with the proliferation data, the cells grown with the GMs/CPCs showed better proliferation ability, appeared much more flattened, and displayed more filopodia than those cells grown with the CPCs. Cell-to-cell interaction could be seen only in the cells with GMs/CPCs. Additionally, the cells could be found as they grew into the pores of the GMs/CPCs (Fig. 5a, b).

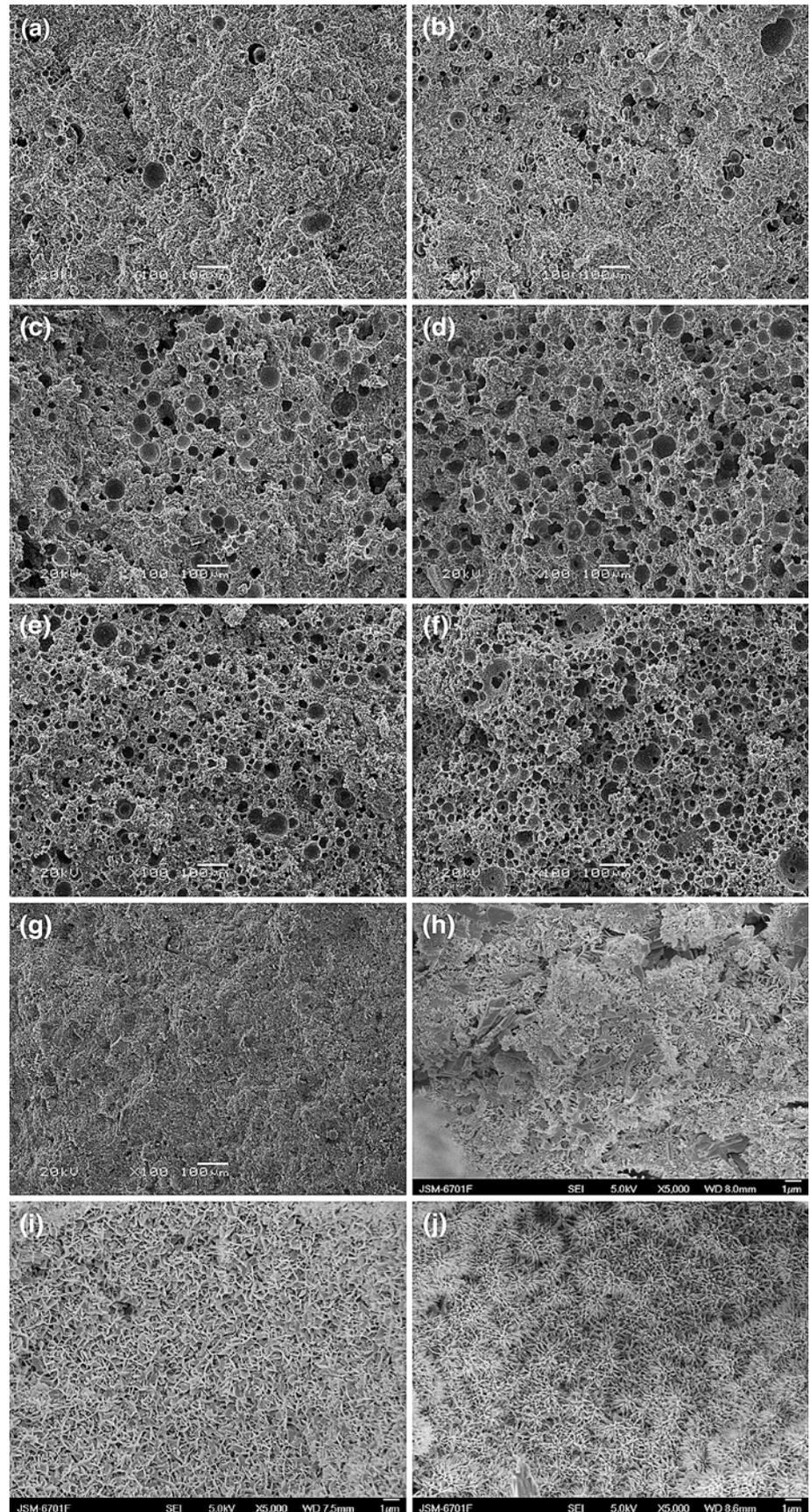
Figure 6 displays the result of the in vivo study. After six weeks, new bones grew into the pores of the GMs/CPCs that resulted from the GM degradation, but there were still some GMs that had not been degraded (Fig. 6b, d, f). There was new bone-growth around the CPCs, but no new bone tissue could be observed inside the composite (Fig. 6a, c, e).

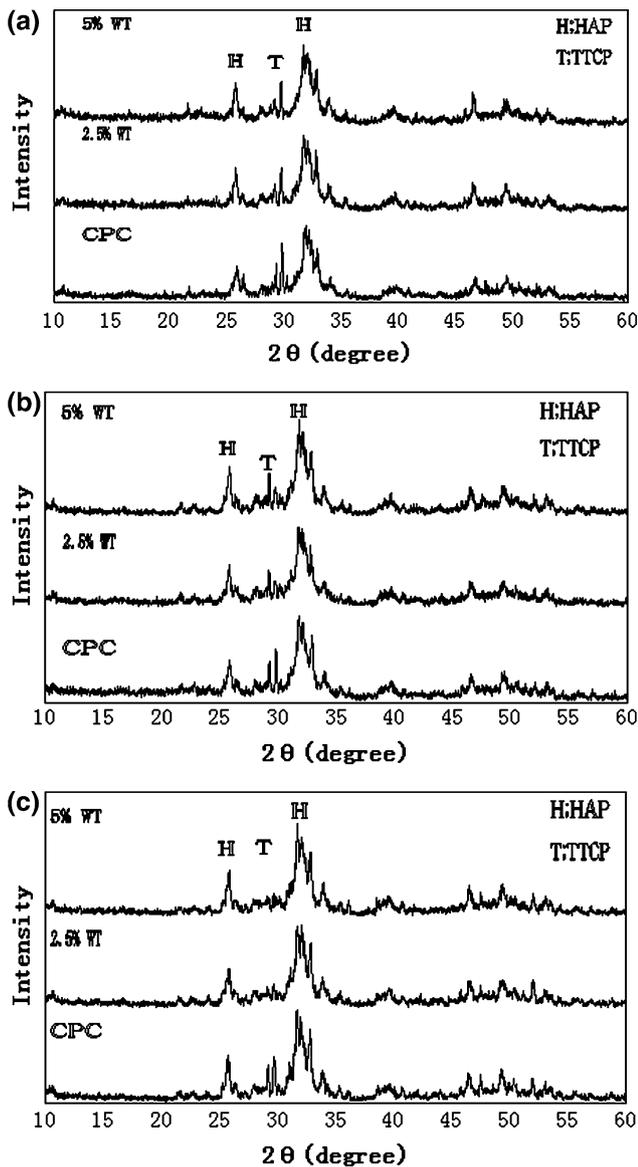
### 4 Discussion

In this study, genipin-crosslinked GMs were incorporated into microporous CPCs to construct a macroporous structure after gelatin degradation. For this purpose, 2.5 wt% and 5 wt% GMs with a 60% degree of crosslinking were added into the CPCs to form two scaffolds of GMs/CPCs. The mechanical and physical characteristics of the tested samples were investigated and compared to microporous CPCs. Furthermore, in vitro and in vivo experiments were performed to evaluate the biocompatibility of the new cement.

It is well known that the setting time always depends on the powder composition, liquid phase, liquid/powder ratio, and ageing conditions [23, 25, 34–36]. Our results demonstrated that the presence of 2.5 wt% and 5 wt% GMs affected the setting time slightly, though it took a little longer time for GMs/CPCs to harden. This finding is concordant with previous studies done with the same liquid/powder ratio. Shie MY et al. [25] confirmed that cements containing uncrosslinked-gelatin took a long time to harden (13 ± 2, 32 ± 1, and 73 ± 2 min for 2, 5, and 10 wt% gelatin, respectively). Bigi et al. [23] also demonstrated that initial and final setting times of CPCs with 18 wt% uncrosslinked-gelatin were 25 min and 45 min, respectively. The reason might be that gelatin may produce polyanions when dissolving, and the excessive amount of

**Fig. 3** Surface SEM micrographs of the 2.5% (a, c, e) and 5 wt% GMs/CPCs (b, d, f) after 1 week (a, b), 3 weeks (c, d), and 5 weeks (e, f) of PBS soaking. Surface SEM micrographs of the CPCs after 5 weeks (g). High power micrographs of CPCs (h), 2.5% (i) and 5 wt% GMs/CPCs (j)





**Fig. 4** XRD patterns of the samples after soaking at different time intervals. **a** 1 week, **b** 3 weeks, **c** 5 weeks

**Table 2** OD values after culturing for different periods

Tested group	Time point (day)		
	One	Three	Five
CPCs control	0.09 ± 0.01	0.26 ± 0.04	0.38 ± 0.05
2.5 wt% GMs\CPCs	0.14 ± 0.02	0.36 ± 0.03	0.50 ± 0.04
TCPS	0.15 ± 0.02	0.37 ± 0.04	0.48 ± 0.05

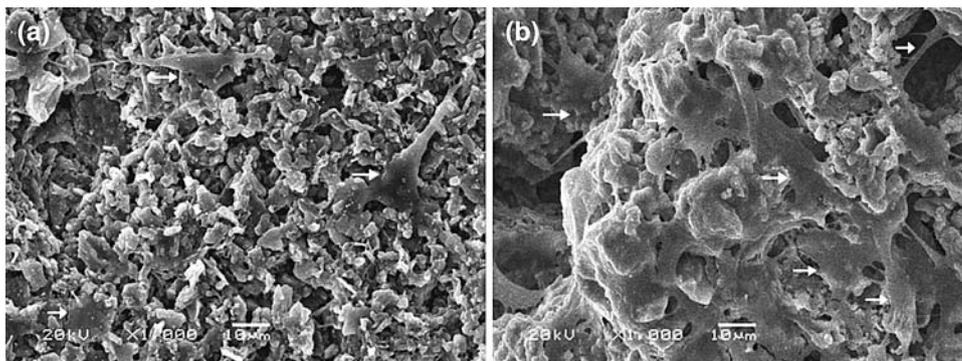
polyanions could destroy the balance of the CPC formulation, leading to a very slow setting process or no setting at all [25]. However, the crosslinked gelatin in our study dissolved very little in the liquid, which produced a relatively small quantity of polyanion in the process of setting.

Therefore, the setting time of our composites was not affected extensively.

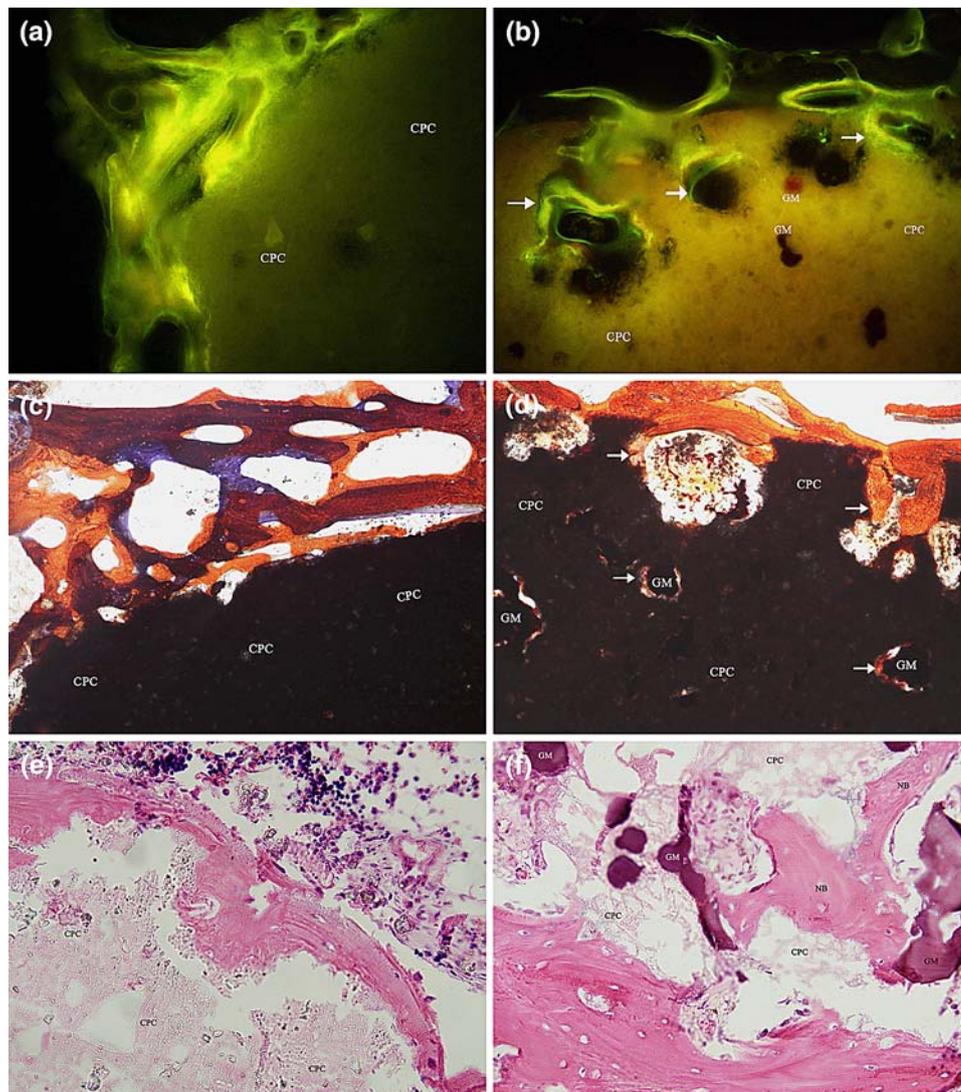
To develop macroporous CPCs, the incorporation of PLGA microspheres into CPCs was reported to be an effective method [9, 17–21]. Gelatin, which has excellent biocompatibility and gradually degrades just like PLGA [26–29], can accelerate the setting reaction and strengthen the mechanical property of CPCs [22, 23]. Therefore, it has potential as an additive material for the introduction of macropores into CPCs. Our finding demonstrated that after three to five weeks of PBS soaking, interconnective porosity could be obtained in both GMs/CPCs. In contrast, with a similar experimental design, Habraken et al. [31] failed to generate a macroporous CPCs by using cross-linked-gelatin. This contradiction most likely resulted from the different degrees of crosslinking of the GMs; we used GMs with a 60% crosslinking degree, which would be absorbed after twenty-eight days in the muscles [28]. Gelatin type was also responsible for the speed of degradation. Type B gelatin, which was used in the study, would degrade faster than the food grade gelatin that has been used by Habraken et al. [31]. Additionally, the degradation of GMs could partly be prevented when they were incorporated into the cement. In our study, some GMs were still present after five weeks of soaking, and many GMs could have been found in the samples achieved from animals. Thus, further experiments are necessary to determine the most favorable crosslinking degree of GMs in the composite. Pore size and interconnective porosity are also two important factors for macroporous CPCs. Because an effective bone in-growth can occur only in pores with a diameter than 50 μm, [37] and with the appropriate interconnectivity, bone tissues can reach the entire material [9, 10, 15, 18]. GMs used in the present study had an average diameter around 50 μm. When mixed with the setting solution, the microspheres swelled and became larger. The size of GMs was enough for bone in-growth. Because cells were found as they grew into the pores of GMs/CPCs, bone tissues could also be detected in the pores of the composites after six weeks of implantation. Regarding inter-connective porosity, we propose that 2.5 wt% GMs was enough for GMs/CPCs because the interconnective microstructure could be clearly seen in the SEM photo of the composite after five weeks of PBS soaking.

Compressive strength is very important for a bone substitute. It was reported that gelatin could improve the compressive strength of CPCs by accelerating the setting reaction, which was the result of interaction between gelatin chains and mineral ions [22, 23, 25]. As proven in our study, the most favorable amount of gelatin added was not consistent, between 2 wt% and 20 wt% [23–25]. Our study showed that the presence of 5 wt% gelatin improved the compressive strength of the CPCs the most after one week

**Fig. 5** SEM micrographs of rat osteoblasts grown on the CPCs (a) and 2.5 wt% GMs/CPCs (b); arrows indicate the cells



**Fig. 6** Micrographs of CPCs (a, c, e) and 2.5 wt% GMs/CPCs (b, d, f) after six weeks of implantation into goat lumbar vertebrae; arrows indicate the new bone (NB). a, b Fluorescence micros photos, magnification of 100×. c, d Modified ponceau trichrism bone stain, magnification of 50×. e, f HE stain, magnification of 400×



of soaking, but after three and five weeks of soaking, the compressive strength of the 2.5 wt% GMs/CPCs was better than that of the 5 wt% GMs/CPCs. The differences between our data and previous studies might be due to different preparation methods as well as the cement types. Further investigation is needed to clarify the proper amount

of gelatin under different circumstances. We also found that the compressive strength of GMs/CPCs decreased and was less than that of CPCs after three or five weeks of PBS soaking. This change of compressive strength might be due to the high porosity and macroporosity of the composite. Porosity and macroporosity play important roles in

affecting the compressive strength of CPCs, and reduction of porosity and macroporosity often improves the compressive strength [9–11, 23–25]. Therefore, the compressive strength of the materials is contradictory to porosity or macroporosity. Macroporous CPCs, as reported previously, were not strong enough for all applications and could only be used as non-load-bearing substitutes [9–16]. Though GMs/CPCs was stronger than other reported macroporous CPCs with similar porosity [9–12, 15–17], and the decrease in compressive strength after GM degradation could partly be compensated by fast bone growth into the porosity [17], it still was not a scaffold that could be used as a temporary load-bearing device.

Since bone is a composite mainly composed of apatite and collagen, CPC containing bioactive components such as gelatin is a good choice for bone tissue engineering. Previous data [26, 27] already demonstrated that the uncrosslinked-gelatin-enriched cement positively stimulated alkaline phosphatase activity, collagen type I, and osteocalcin production, not only in normal osteoblasts, but also in osteopenic osteoblasts culture. Though the cytotoxicity of genipin is less than that of the other crosslinkers (such as formaldehyde, glutaraldehyde, dialdehyde starch and epoxy compound) [38, 39], there is still a need for further tests to confirm whether the composite with genipin crosslinked GMs and CPCs is biocompatible. To our knowledge, no such data have been reported. Our experiment was the first to demonstrate that the presence of genipin crosslinked GMs did not affect the morphology of osteoblasts but improved their proliferation. Animal studies also showed that new bones could grow into the pores of the composite. As previously reported [28, 39], GMs crosslinked at a 0.44 M concentration of genipin resulted in a mild inflammatory reaction in the animal muscle, and tricalcium phosphate scaffold with 0.5 wt% genipin showed excellent biocompatibility with the subcutaneous tissue of rat. Therefore, with the proper concentration of genipin, CPC with crosslinked GMs appears to be a promising biomimetic material that can be successfully applied as a bone substitute.

## 5 Conclusions

This study indicates that macroporous CPCs can be obtained by using genipin crosslinked GMs. The initial mechanical property of the composite can also be enhanced, but it will decrease after GM degradation. Gelatin microspheres at a mass fraction of 2.5% are optimal for the composite. The new composite demonstrates a good biocompatibility in vitro and in vivo, suggesting that it is a promising candidate as a bone substitute under non-compression-loaded circumstances.

**Acknowledgments** This work was funded by Medical Science Research Foundation of Chinese People's Liberation Army (Grant Number of 06MA090) and Natural Science Foundation of Gansu Province (Grant Number of 0710RJZA068). CPCs were kindly supplied by Shanghai Rebone Biomaterials (China).

## References

1. L. Comuzzi, E. Ooms, J.A. Jansen, *Clin. Oral Implants Res.* **13**, 304 (2002). doi:10.1034/j.1600-0501.2002.130311.x
2. E.M. Ooms, J.G. Wolke, J.P. van der Waerden, J.A. Jansen, *J. Biomed. Mater. Res.* **61**, 9 (2002). doi:10.1002/jbm.10029
3. M.R. Sarkar, N. Wachter, P. Patka, L. Kinzl, *J. Biomed. Mater. Res.* **58**, 329 (2001). doi:10.1002/1097-4636(2001)58:3<329::AID-JBM1025>3.0.CO;2-9
4. R.Z. LeGeros, J.R. Parsons, G. Daculsi, F. Driessens, D. Lee, S.T. Liu, S. Metsger, D. Peterson, M. Walker, *Ann. NY Acad. Sci.* **523**, 268 (1988). doi:10.1111/j.1749-6632.1988.tb38519.x
5. L.C. Chow, *Monogr. Oral Sci.* **18**, 148 (2001). doi:10.1159/000061653
6. M.F. Baslé, D. Chappard, F. Grizon, R. Filmon, J. Delecric, G. Daculsi, A. Rebel, *Calcif. Tissue Int.* **53**, 348 (1993). doi:10.1007/BF01351842
7. C.M. Clokie, H. Moghadam, M.T. Jaskson, G.K. Sandor, *J. Craniofac. Surg.* **13**, 111 (2002). doi:10.1097/00001665-200201000-00026
8. H. Schliephake, R. Grubber, M. Dard, R. Wenz, S. Scholz, *J. Biomed. Mater. Res. A* **69**, 382 (2004). doi:10.1002/jbm.a.20121
9. P.Q. Ruhé, E.L. Hedberg-Dirk, N.T. Padron, P.H. Spauwen, J.A. Jansen, A.G. Mikos, *Tissue Eng.* **12**, 789 (2006). doi:10.1089/ten.2006.12.789
10. R.P. del Real, E. Ooms, J.G. Wolke, M. Vallet-Regí, J.A. Jansen, *J. Biomed. Mater. Res. A* **65**, 30 (2003). doi:10.1002/jbm.a.10432
11. S. Takagi, L.C. Chow, *J. Mater. Sci. Mater. Med.* **12**, 135 (2001). doi:10.1023/A:1008917910468
12. Y. Zhang, H.H. Xu, S. Takagi, L.C. Chow, *J. Mater. Sci. Mater. Med.* **5**, 437 (2006). doi:10.1007/s10856-006-8471-z
13. H.H. Xu, M.D. Weir, E.F. Burguera, A.M. Fraser, *Biomaterials* **24**, 4279 (2006). doi:10.1016/j.biomaterials.2006.03.001
14. H.H. Xu, L.E. Carey, C.G. Simon Jr, Simon, *J. Mater. Sci. Mater. Med.* **18**, 1345 (2007). doi:10.1007/s10856-007-0146-x
15. S. Hesaraki, F. Moztaazadeh, D. Sharifi, *J. Biomed. Mater. Res. A* **83**, 80 (2007). doi:10.1002/jbm.a.31196
16. S. Sarda, M. Nilsson, M. Balcells, E. Fernandez, *J. Biomed. Mater. Res. A* **65**, 215 (2003). doi:10.1002/jbm.a.10458
17. D.P. Link, J. van den Dolder, W.J. Jurgens, J.G. Wolke, J.A. Jansen, *Biomaterials* **27**, 4941 (2006). doi:10.1016/j.biomaterials.2006.05.022
18. W.J. Habraken, J.G. Wolke, A.G. Mikos, J.A. Jansen, *J. Biomater. Sci. Polym. Ed.* **17**, 1057 (2006). doi:10.1163/156856206778366004
19. E.W. Bodde, O.C. Boerman, F.G. Russel, A.G. Mikos, P.H. Spauwen, J.A. Jansen, *J. Biomed. Mater. Res. A* [Jan 15, Epub ahead of print] (2008)
20. Z. Fei, Y. Hu, D. Wu, H. Wu, R. Lu, J. Bai, H. Song, *J. Mater. Sci. Mater. Med.* **19**, 1109 (2008). doi:10.1007/s10856-007-3050-5
21. P.Q. Ruhé, O.C. Boerman, F.G. Russel, P.H. Spauwen, A.G. Mikos, J.A. Jansen, *J. Control Release* **106**, 162 (2005). doi:10.1016/j.jconrel.2005.04.018
22. Z. Pan, P. Jiang, Q. Fan, B. Ma, H. Cai, *J. Biomed. Mater. Res. B Appl. Biomater.* **82**, 246 (2007). doi:10.1002/jbm.b.30727

23. A. Bigi, B. Bracci, S. Panzavolta, *Biomaterials* **25**, 2893 (2004). doi:[10.1016/j.biomaterials.2003.09.059](https://doi.org/10.1016/j.biomaterials.2003.09.059)
24. Y. Fujishiro, K. Takahashi, T. Sato, *J. Biomed. Mater. Res.* **54**, 525 (2001). doi:[10.1002/1097-4636\(20010315\)54:4<525::AID-JBM80>3.0.CO;2-#](https://doi.org/10.1002/1097-4636(20010315)54:4<525::AID-JBM80>3.0.CO;2-#)
25. M.Y. Shie, D.C. Chen, C.Y. Wang, T.Y. Chiang, S.J. Ding, *Acta Biomater.* **4**, 646 (2008). doi:[10.1016/j.actbio.2007.10.011](https://doi.org/10.1016/j.actbio.2007.10.011)
26. A. Bigi, P. Torricelli, M. Fini, B. Bracci, S. Panzavolta, L. Sturba, R. Giardino, *Int. J. Artif. Organs* **27**, 664 (2004)
27. A. Bigi, S. Panzavolta, L. Sturba, P. Torricelli, M. Fini, R. Giardino, *J. Biomed. Mater. Res. A* **78**, 739 (2006). doi:[10.1002/jbm.a.30765](https://doi.org/10.1002/jbm.a.30765)
28. H.C. Liang, W.H. Chang, K.J. Lin, H.W. Sung, *J. Biomed. Mater. Res. A* **65**, 271 (2003). doi:[10.1002/jbm.a.10476](https://doi.org/10.1002/jbm.a.10476)
29. H.J. Wei, H.H. Yang, C.H. Chen, W.W. Lin, S.C. Chen, P.H. Lai, Y. Chang, H.W. Sung, *J. Control Release* **120**, 27 (2007). doi:[10.1016/j.jconrel.2007.04.005](https://doi.org/10.1016/j.jconrel.2007.04.005)
30. M.I. Ugwoke, R. Kinget, *J. Microencapsul.* **15**, 273 (1998). doi:[10.3109/02652049809006857](https://doi.org/10.3109/02652049809006857)
31. W.J. Habraken, L.T. de Jonge, J.G. Wolke, L. Yubao, A.G. Mikos, J.A. Jansen, *J. Biomed. Mater. Res. A* [Jan 11, Epub ahead of print] (2008)
32. L.E. Carey, H.H. Xu Jr, C.G. Simon, S. Takagi, L.C. Chow, *Biomaterials* **26**, 5002 (2005). doi:[10.1016/j.biomaterials.2005.01.015](https://doi.org/10.1016/j.biomaterials.2005.01.015)
33. W.C. Vroouwenvelder, G.G. Groot, K. de Groot, *Biomaterials* **13**, 382 (1992). doi:[10.1016/0142-9612\(92\)90044-O](https://doi.org/10.1016/0142-9612(92)90044-O)
34. M. Kon, Y. Miyamoto, K. Asaoka, K. Ishikawa, H.H. Lee, *Dent. Mater. J.* **17**, 223 (1998)
35. E. Fernández, F.J. Gil, M.P. Ginebra, F.C. Driessens, J.A. Planell, S.M. Best, *J. Mater. Sci. Mater. Med.* **10**, 223 (1999). doi:[10.1023/A:1008958112257](https://doi.org/10.1023/A:1008958112257)
36. H. el-Briak, D. Durand, J. Nurit, S. Munier, B. Pauvert, P. Boudville, *J. Biomed. Mater. Res.* **63**, 447 (2002). doi:[10.1002/jbm.10257](https://doi.org/10.1002/jbm.10257)
37. A.I. Itälä, H.O. Ylänen, C. Ekholm, K.H. Karlsson, H.T. Aro, *J. Biomed. Mater. Res.* **58**, 679 (2001). doi:[10.1002/jbm.1069](https://doi.org/10.1002/jbm.1069)
38. H.W. Sung, R.N. Huang, L.L. Huang, C.C. Tsai, C.T. Chiu, *J. Biomed. Mater. Res.* **42**, 560 (1998). doi:[10.1002/\(SICI\)1097-4636\(19981215\)42:4<560::AID-JBM12>3.0.CO;2-I](https://doi.org/10.1002/(SICI)1097-4636(19981215)42:4<560::AID-JBM12>3.0.CO;2-I)
39. C.H. Yao, B.S. Liu, S.H. Hsu, Y.S. Chen, C.C. Tsai, *J. Biomed. Mater. Res. A* **69**, 709 (2004). doi:[10.1002/jbm.a.30045](https://doi.org/10.1002/jbm.a.30045)