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Development of a chitosan–polyglutamate based injectable polyelectrolyte complex scaffold

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ABSTRACT

In this study, the chitosan–polyglutamate hydrogel was developed as injectable polyelectrolyte complex (PEC) scaffold for bone regeneration. In practice, oppositely charged polyelectrolytes were mixed using a static injection mixer, the gelation time was less than 1 min. The resorbable PEC scaffold can be in situ-formed as a scaffold for osteoblast ingrowth. The canine alveolar defect model was used to evaluate the efficacy of PEC implants. The PEC scaffold was highly effective in enhancing bone regeneration. Moreover, the addition of $CaSO_4 \cdot 2H_2O$ (CSD) to PEC scaffold can further enhance the early stage healing of bone regeneration.

1. Introduction

Bone grafts are commonly used in orthopedics and dentistry to repair bony defects due to trauma, disease, or surgery (Taylor, 1983). Autograft bone is most effective for bone repair; however, the supply of autogenous bone grafts is limited. In addition, allografts are susceptible to immune responses and disease transmission. Therefore, numerous synthetic bone substitutes have been used in clinical practice because of their biodegradation, biocompatibility, and osteoconductivity. However, it is difficult for prefabricated bone blocks to fit an irregular bony defect (Xu, Weir, Burguera, & Fraser, 2006). Although granular-type bone grafts can easily be filled into an irregular defect, it is difficult to maintain them in defect sites, especially with incomplete bony defects (Yao, Liu, Hsu, & Chen, 2005). To overcome these problems, injectable scaffolds are highly desirable in clinical applications (Wintermantel et al., 1996). After injection, a precursor solution can fill in an irregular defect, and the gel can form in situ. In addition, injectable scaffolds can also serve as a carrier for cells and/or bioactive molecules applied via minimally invasive procedures (Kretlow, Klouda, & Mikos, 2007).

Injectable scaffolds are formed via an in situ crosslinking method, which is a critical technique in developing, as it affects the character of the resulting gel (e.g., the gelling time, gel content, porosity, degradation time, and biocompatibility) (Yu & Ding, 2008). Commonly used in situ crosslinking methods are classified into chemical and physical types. Chemical crosslinking (redox- or photo-initiated) are controlled by adjusting the initiator concentration or radiation dosage to obtain a desirable gelling time and degradability. However, chemical crosslinkers are usually toxic, which is a major obstacle to their applications (Hou, De Bank, & Shakesheff, 2004). In comparison, physical ionic crosslinking displays much fewer biocompatibility concerns. For this reason, an ionically crosslinked polyelectrolyte complex (PEC) method was chosen for this study. The PEC consisted of a complex of oppositely charged (cationic and anionic) macromolecules that were bound by electrostatic interactions (Li et al., 2007).

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Chitosan is a polysaccharide constituted by N-glucosamine and N-acetyl-glucosamine units, and it has been widely investigated for bone regeneration (Muzzarelli, 2009, 2011). Polyglutamate (PG), a natural polyanionic peptide produced by *Bacillus subtilis*, is a hydrophilic, biocompatible, and biodegradable material that has been successfully used in medical applications (Richard & Margaritis, 2001). A chitosan–PG was used to fabricate hydrogels, and preformed scaffolds in several recent studies (Hsieh, Tsai, Wang, Chang, & Hsieh, 2005; Tsao et al., 2010). However, no report has so far used a chitosan–PG hydrogel to develop injectable scaffold for bone regeneration. In this study, the PEC structure formed was mainly based on electrostatic interactions of chitosan and PG. In order to improve the osteogenic potential of the designed PEC, calcium sulfate dihydrate (CSD) was added. CSD acts at a preribosomal level and regulates the translation process to increase the expression of bone-related proteins (Palmieri et al., 2008). Lazary et al. (2007) also reported that CSD is a passive osteoconductive material, and it might also have potential osteoinductivity due to its high calcium ion release. For these reasons, incorporation of CSD into a PEC system may be a good combination to enhance new bone formation.

In the process of forming the PEC, it was difficult to achieve homogeneity of the hydrogel by a direct mixing method. During mixing the two oppositely charged polyelectrolytes with a magnetic stirrer, the PEC usually formed at the interfaces as huge aggregates (Hsieh et al., 2005). To develop an in situ-formed scaffold, the two oppositely charged polyelectrolytes had to be mixed during the injection period. The static mixer is widely used in a range of applications such as for the continuous mixing of viscous liquids, blending, and chemical reactions (Paul, Atiemo-Obeng, & Kresta, 2004). Thus, the static mixing system was chosen as the injection mixer in this experiment.

The aims of this study were to develop a non-toxic PEC formulation which can be injected into defects and solidified in situ with a static injection mixer; moreover, CSD was added to the PEC to provide adequate calcium ions to enhance bone regeneration. The osteogenetic ability of the designed specimens was evaluated in a canine alveolar defect model using histological and histomorphometric methods.

2. Materials and methods

2.1. Materials

Chitosan (MW 25,000–35,000) with a degree of deacetylation of 95% was obtained from Shin Era Technology (Taipei, Taiwan). Polyglutamate (PG, MW 1000 kDa) was purchased from Vedan (Taipei, Taiwan). Phosphate-buffered saline (PBS), potassium bromide, high-viscosity carboxy-methyl-cellulose (CM-cellulose), and MTT were purchased by Sigma (St. Louis, MO, USA). Calcium sulfate dihydrate (CSD) was purchased from Biotech One (Taipei, Taiwan). The decalcifying agent was purchased from Surgipath Medical Industries (Richmond, IL, USA). Trypsin-EDTA, α-minimum essential medium (MEM), and fetal bovine serum (FBS) were purchased from Gibco BRL Life Technology (Grand Island, NY, USA).

2.2. Preparation of specimens (PEC and PEC/CSD)

Chitosan was dissolved in 1% acetic acid, and the final pH of the solution was adjusted to 6.2. PG and CM-cellulose were dissolved in double-distilled water (DDW), and the pH of the solution was adjusted to 7.5 to neutralize the cationic polyelectrolytes. The results of the following gelation experiments were used to formulate and characterize the PEC. When preparing the PEC/CSD group, CSD was added to the polycationic and polyanionic solutions, respectively. The final concentration of CSD in the PEC system was 20% (w/w). The cationic and anionic polyelectrolytes were mixed in a 1:1 (v/v) ratio with a static injection mixer (Sulzer, Salem, NH, USA) at room temperature.

2.3. Analytical determination of PEC

In the gelling time test, 2 ml of PEC was injected into a glass beaker with a 5 ml capacity which contained a stirring bar (with a diameter of 5 mm and a length of 10 mm) at room temperature, and then placed on a stirring plate (Corning, Painted Post, NY, USA) at a stirring speed of 45 rpm. The gelling time was noted as the time required for the stirring bar to stop (Balakrishnan & Jayakrishnan, 2005; Hong, Mao, Wang, Gao, & Shen, 2006).

In the gel content test, specimens were lyophilized and weighed. Acetic acid (1%; a solvent for chitosan, PG, and CM-cellulose) was used to extract non-crosslinked material from the specimen. The gel content was calculated from the dry weight of a specimen before and after solvent extraction (Lee, Hung, Cheng, & Wang, 2005). All test specimens were extracted in the solvent for 24 h, washed with DDW, dried in an oven at 80 °C, and then weighed. The gel content was calculated using the equation:

$$\text{gel content}\,(\%) = \frac{W_a}{W_b} \times 100;$$

where W_b and W_a are the dry weights of the specimens before and after extraction, respectively.

To analysis the equilibrium swelling, specimens were injected into the wells of culture plates (12-well) and dried in a freeze-drier. The dry weights (W_d) were immediately measured, and then the specimens were immersed in PBS, maintained at 37 °C for 24 h, and weighed to obtain the wet weight (W_w). The equilibrium swelling (ES) of the matrices was calculated using the following equation:

$$\text{equilibrium swelling value} = \frac{W_w - W_d}{W_d}.$$

To examine the morphology of the PEC, cross-sections of the lyophilized PEC were directly sputter-coated with gold. Microstructures of the lyophilized specimen and cell-containing specimen were examined by SEM (Hitachi S2400, Tokyo, Japan) under an accelerating voltage of 15–20 kV.

In vitro degradation test was conducted according to ISO10993-9 guidelines; specimens were incubated in 50 ml of PBS at 37 °C with agitation at 50 rpm. After 1, 2, 4, 12, and 26 weeks of incubation, specimens were lyophilized. The amount of degradation was calculated using the following equation:

$$\text{weight retention}\,(\%) = \frac{W_t}{W_d} \times 100;$$

where W_d is the weight of the initial dry specimen, and W_t is the weight of the dry specimen at time t.

2.4. In vitro cell compatibility and in vivo implantation test

In accordance with ISO10993-5 guidelines, the cell compatibility of the PEC was evaluated on MC3T3E1 cell (pre-osteoblast) monolayer using the MTT method. The extracted medium for testing was prepared by incubating the specimen with 10% FBS at an extraction ratio of 0.2 g/ml for 24 h at 37 °C. Extracted medium of the PEC (experimental group), extracted medium of high-density polyethylene (negative control, NC), medium with 0.5% DMSO (positive control, PC), and normal culture medium (control, C) were placed on a monolayer of cells. After incubation at 37 °C for 24 h, the cellular responses were assessed by optical microscopy and the MTT assay. The absorbance of MTT in each well was immediately recorded at 570 nm using a UV/visible absorbance spectroscopy

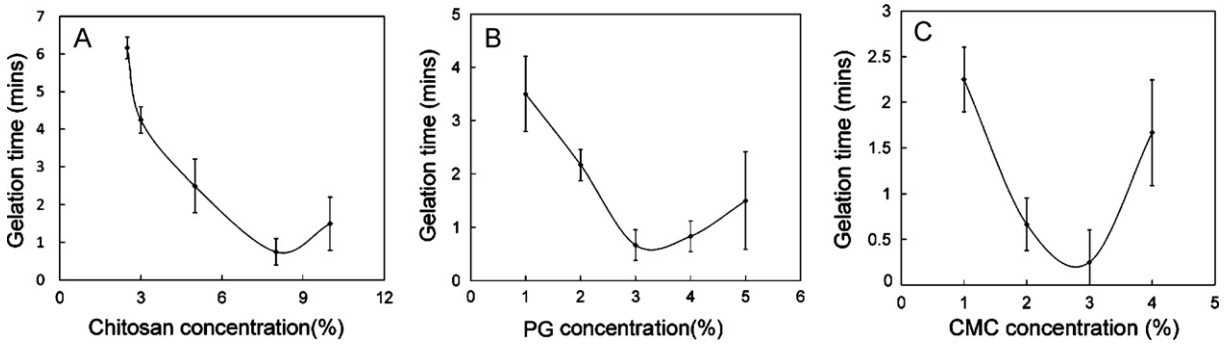

Fig. 1. Gelation times at different concentrations (A) chitosan (mixed with 3% PG + 2% CM-cellulose), (B) PG + 2% CM-cellulose (mixed with 8% chitosan), and (C) CM-cellulose + 3% PG (mixed with 8% chitosan) at 25 °C ($n = 4$).

(Bio-Tek Instruments, Winooski, VT, USA). The cell compatibility was estimated using the following equation:

$$\text{cell viability (\% of control)} = \frac{\text{absorbance of the test material}}{\text{absorbance of the control}} \times 100.$$

In the cell attachment test, the PEC was sterilized in 75% ethanol for 24 h. After washing with PBS, 5000 MC3T3E1 cells were seeded on a gel and incubated at 37 °C in an incubator with a 0.5% CO_2 atmosphere for 72 h. Cell-containing specimens were fixed with a 1 wt% glutaraldehyde solution and 1 wt% OsO_4 (post-fixed). Specimens were dehydrated in a graded ethanol series (30, 50, 70, 80, 90, and 100 wt%), and then dried by critical-point drying with liquid CO_2. Finally, cells containing PEC were coated with gold and observed by SEM.

The canine alveolar bone defect implantation test was performed in strict accordance with protocols approved by the Animal Care Committee of Taipei Medical University. Eight beagles with a mean age of 1 year were operated on under general anesthesia. All pre-molars in the mandible were extracted. When the extraction socket had healed (about 2 months), six defects (6 mm in depth and 6 mm in diameter) on both sides of the mandible were created with a trephine bur. The defects were filled with the following graft materials: PEC, PEC/CSD, and CSD (particle size 500–1000 μm). The same procedure was performed in the empty control group. To identifying the location of the defect, we recorded the distance between the canine teeth and each defect on alveolar ridge. Specimens were harvested after 3 and 6 weeks. Each implantation site was harvested with a trephine bur (with an inner diameter of 6 mm) at 3 and 6 weeks after implantation. The harvested specimens were fixed in 10% buffered formalin for 1 week, and a cross-section of the specimen was created with a low-speed cutter (Techcut 4^{TM}, Allied High Tech Products, Rancho Dominguez, CA, USA), and then the specimen was decalcified until the specimen could be penetrated with a needle. Finally, the decalcified sample was prepared to a paraffin section, and stained with hematoxylin and eosin (H&E). The stained section was observed under light microscopy (Olympus EX51, Tokyo, Japan). In this study, histomorphometric method was used to quantify the amount of newly formed bone tissue within the grafted area, which was carried out with an optical microscope connected to a personal computer equipped with an image analysis system. The defect boundaries and cavity area were visually determined, and all areas of the image were quantified using Image J analysis software (National Institutes of Health, Bethesda, MD, USA). The amount of newly formed bone was calculated using following equation (Park, Jang, Bae, An, & Suh, 2009):

$$\text{new bone (\%)} = \frac{\text{area of regenerated bone}}{\text{area of created defect}} \times 100.$$

2.5. Statistical analysis

Group means and standard deviations (SD) were calculated for each measured parameter. Data were compared using a one-way analysis of variance (ANOVA) and post hoc Tukey's test, and p values of <0.05 indicated statistical significance.

3. Results and discussion

3.1. Analytical determination of PEC (gelation property, micromorphology and degradation)

Gelation times of the specimens were estimated at room temperature. The concentrations of chitosan, PG, and CM-cellulose were respectively controlled to 2.5–10%, 1–5%, and 1–5%, because of operative feasibility (e.g., solubility and viscosity). In our system, gelation times of specimens were between 40 s and 6 min (Fig. 1).

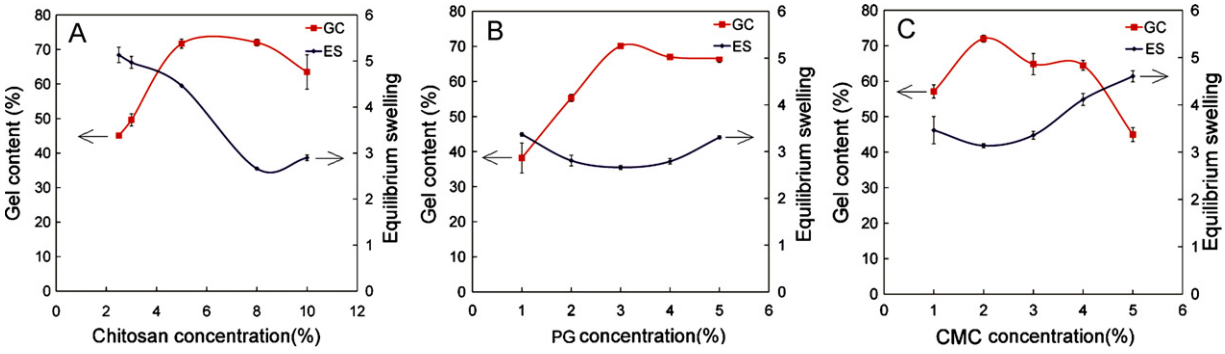

Fig. 2. Gel content (GC) (♦) and equilibrium swelling (ES) at 25 °C in PBS for 24 h (■) of complexes formed by mixing different concentrations of (A) chitosan (mixed with 3% PG + 2% CM-cellulose), (B) PG + 2% CM-cellulose (mixed with 8% chitosan), and (C) CM-cellulose + 3% PG (mixed with 8% chitosan) ($n = 4$).

The gelation time could be adjusted by changing the concentration of the polymer; however, the tendency of the gelation time did not seem to be positively correlated with the polymer concentration. At the respective concentrations of chitosan, PG, and CM-cellulose of 8%, 3%, and 2% (w/w), the specimen had the fastest gelation time (40 s). As an injectable scaffold system, an appropriate gelling time is important; it is known that the setting times of commercial injectable calcium phosphate cement (CPC) systems are about 10–30 min, which usually cause the CPC to be wash-out with body fluids during operation (Xu & Simon, 2005). Generally, chemical and ionic crosslinking injectable scaffold systems usually have less solidification periods compared to commercial CPC systems. Our PEC system can gel within 1 min that is similar to Tan's modified chitosan–hyaluronic acid gel system (about 1 min) and Jin's chitosan derivative enzymatic crosslinked gel system (10 s) (Jin et al., 2009; Tan, Chu, Payne, & Marra, 2009).

The gel contents were related to the number of materials forming the PEC structure. Fig. 2 displays the gel contents which were not positively correlated with the concentrations of materials. The specimen composed of 8% chitosan and 3% PG + 2% CM-cellulose had the maximum gel content (75%), this indicates that PEC formation is more efficient at nearly a stoichiometric composition. The swelling ratio is an important parameter to characterize crosslinked structure. The effect of the polymer concentration on swelling is shown in Fig. 2. The equilibrium swelling values of the designed specimens were within 2.7–5.1. There was no linear relationship between the concentrations of materials and the swelling values. The swelling value was inversely proportional to the gel content. The gel content of the PEC was associated with the swelling behavior; the higher gel content resulted in a stronger crosslinked structure which confines the macromolecules to a limited spatial volume, so the swelling value is largely restricted. Sumi, Kala, Praveen, and Prasada Rao (2008) reported that a low swelling value was associated with a low drug release rate. Moreover, environmental changes in pH and temperature also affect the swelling behavior (Karg et al., 2008). According to the above results, the formulation of 8% chitosan and 3% PG + 2% CM-cellulose had the highest gel content (75%), the lowest equilibrium swelling value (2.7) and the fastest gelling time (40 s), which may have been closest to the stoichiometric formulation and was chosen for the following experiments.

When PEC scaffold was injected into a cylindrical mold with the static injection mixer, after 24 h, the specimen showed a good ability to maintain its shape due to physical crosslinking (Fig. 3A). According to the cross-sectional SEM images (Fig. 3B and C), the designed PEC had an interconnected porous structure. Pore sizes were around 100–500 μm and the surface microstructures were very rough. In the microstructure of the PEC (Fig. 3B), the interconnected pore structure may support a biological environment conducive to cell attachment and proliferation; moreover, a suitable interconnected porous structure can allow regenerated tissue ingrowth and the exchange of nutrients (Li, Ramay, Hauch, Xiao, & Zhang, 2005), Moreover, the rough surface microstructure (Fig. 3C) was suitable for cell adhesion (Fig. 5C) (Citeau et al., 2005).

The weight loss of the PEC in PBS at 37 °C was measured over time. Fig. 4 indicates that the weight loss was very fast in the first week (27.7% weight loss); afterwards, the degradation rate decreased. During the period of 1–26 weeks, the weight loss of PEC in PBS was 24.9%, the total weight loss of the PEC was 47.4% at 26 weeks, and the shape of the PEC was still intact; thus the designed PEC crosslinked structure had high stability within 26 weeks. Generally, the stability of ionic bonding is weaker than chemical covalent bonding which is related to the degradation rate. Balakrishnan and Jayakrishnan (2005) reported an oxidized alginate-based self-crosslinking gel, which degraded by about 50% within 5 weeks. According to Tan's report, a modified

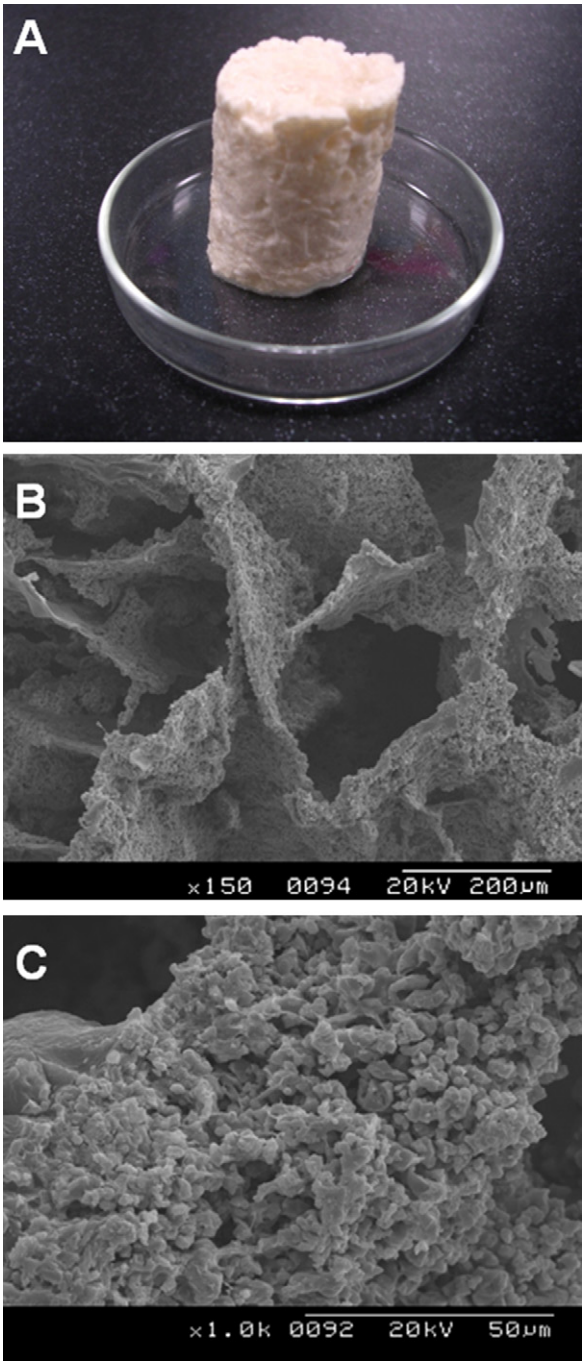

Fig. 3. (A) PEC (injected into a mold). (B) Fine structure of the lyophilized PEC (magnification, 150×). (C) Surface fine structure of the lyophilized PEC (magnification, 1000×).

chitosan–alginate gel system showed a degradation of 38.6% within 28 days (Jin et al., 2009). In this study, the degradation of PEC was about 36.7% at 28 days which was similar to the above-mentioned chemically modified gel, and the in vitro degradation of PEC was 48.4% after 26 weeks (Fig. 4).

3.2. Biocompatibility and canine implantation test

According to ISO10993-5 guidelines, cell compatibility was evaluated in MC3T3E1 pre-osteoblasts by the MTT assay. As shown in Fig. 5A, the PEC extract induced no cell morphological changes (e.g., shrinkage or lysis). The MTT results of the control, negative

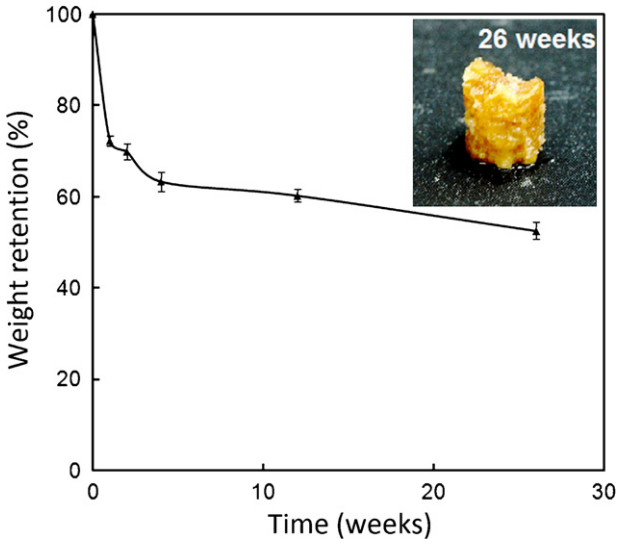

Fig. 4. Degradation profile of the PEC at 26 weeks in PBS at 37 °C ($n = 3$).

control, and PEC groups showed that there were no statistically significant differences, which revealed that the PEC had little cytotoxicity (Fig. 5B). Moreover, values of the positive and negative controls were 41.4% and 98.7%, respectively, which proves that the tests were done correctly. SEM photographs (Fig. 5C) showed that MC3T3E1 cells had adhered to the material surface, and the pseudopodia were well extended. Thus, this PEC system may be able to provide sufficient stability and satisfactory biocompatibility compared to traditional chemical crosslinking methods.

In animal study, specimens (PEC, PEC/CSD, and CSD) were implanted in the canine alveolar bone defects and harvested at 3 and 6 weeks postoperatively. The bone-regenerating efficacy was evaluated by histological and histomorphometric methods. Histological pictures are given in Fig. 6. The empty control group was filled with connective tissue, and a similar result was displayed in the CSD group, both of which showed little bone formation after 3 weeks. However, in the PEC group, some new bone had formed at the defect edges, while connective tissue still existed in the center of the defect. In the PEC/CSD group, a substantial amount of new bone formation was observed within 3 weeks, but there was still a cavity in the central part that may have been caused by unresorbed material. Six weeks postoperatively, some new bone was observed in the empty control, and Fig. 6D3 showed the entire left part of the defect in the CSD group was filled with connective tissue; this may have been due to loss of the graft. If the walls of the

Table 1
Mean percentage of new bone formed (empty control, PEC, PEC/calcium sulfate dihydrate (CSD), and CSD (granules)) in a canine alveolar defect model, each groups were compared using a one-way analysis of variance (ANOVA) and post hoc Tukey's test ($n = 4$) and p values of <0.05 indicated statistical significance[a].

Group	New bone formation	
	3 weeksMean ± SD (%)	6 weeksMean ± SD (%)
Empty control	0.5 ± 0.3[a]	41.9 ± 9.5[a]
PEC	22.7 ± 7.3[b]	68.7 ± 12.8[b]
PEC/CSD	50.1 ± 16.5[c]	71.6 ± 5.9[b]
CSD (granule)	9.1 ± 7.9[a,b]	42.4 ± 8.9[a]

[a] Different letters (a–c) in the same column denote a significant difference ($p < 0.05$).

defect were incomplete, it would be hard to contain the granular graft in the defect to support osteoprogenitor cell growth and new bone growth (Tischler & Misch, 2004). But by then, the defects filled with PEC and PEC/CSD had almost completely healed. Slight immune or inflammatory responses were evident at all implant sites based on the histological evaluation. New bone formation was quantified by an image analysis, and histomorphometric results are summarized in Table 1. Three weeks postoperatively, percentages of new bone formation in the empty control, PEC, PEC/CSD, and CSD groups were 0.5% ± 0.3%, 22.7% ± 7.3%, 50.1% ± 16.5%, and 9.1% ± 7.9%, respectively; hence, defects filled with the PEC/CSD healed at a notably faster rate than did the other defects. Six weeks postoperatively, levels of new bone formation in the empty control and CSD groups were 41.9% ± 9.5% and 42.4% ± 8.9%, respectively, with no significant difference. In the PEC and PEC/CSD groups, extensive new bone was observed after 6 weeks, and there was no significant difference in the percentages of new bone formation (68.7% ± 12.8% and 71.6% ± 5.9%, respectively). We compared the PEC with PEC/CSD to estimate the efficacy of adding CSD to the PEC system. According to the histomorphometric results (Table 1), adding CSD to the PEC enhanced bone regeneration, especially in the early stage. The extracellular Ca^{2+} concentration at the site of bone remodeling reached up to 40 mM, and the moderately high extracellular Ca^{2+} concentrations enhanced osteoblast differentiation (Nakade, Takahashi, Takuma, Aoki, & Kaku, 2001; Yamaguchi et al., 1998). The solubility of CSD is 14 mM, and thus including CSD in the PEC can increase the extracellular concentration of Ca^{2+}, which may act on a preribosomal level and affects regulation of the translation process to enhance the expression of bone-related proteins (Palmieri et al., 2008). When mesenchymal stem cells or osteocytes migrate into the PEC/CSD, this Ca^{2+} concentration may be able to enhance cell osteogenic differentiation and promote bone regeneration.

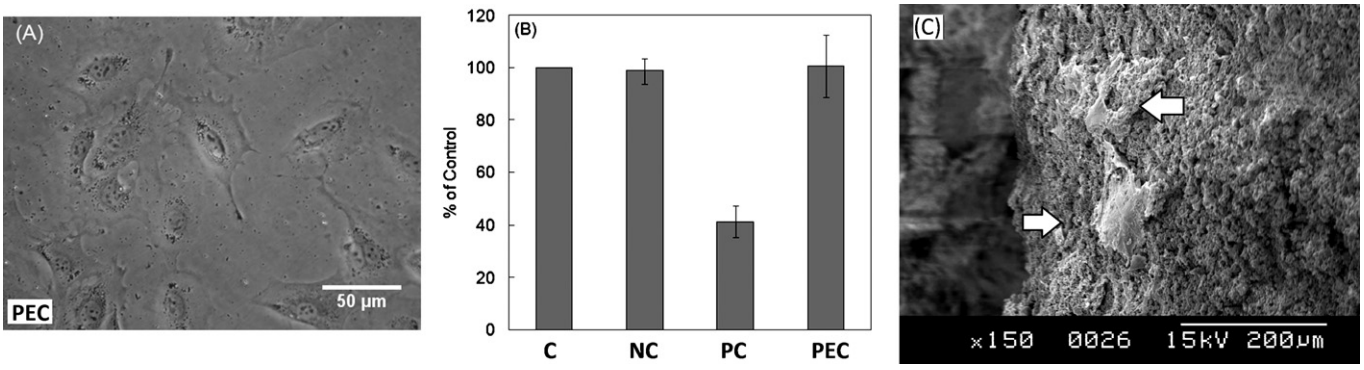

Fig. 5. (A) Optical photomicrograph (100×) of MC3T3E1 osteoblast culture in PEC extract medium. (B) MTT reduction by MC3T3E1 cells exposed to the PEC extract compared to MTT reduction in the control (cells with medium alone), negative control (cells with high-density polyethylene), and positive control (cells with 0.5% DMSO) for 24 h. (C) Scanning electron micrographs showing the growth of MC3T3E1 cells on the PEC for 3 days (magnifications, 150×).

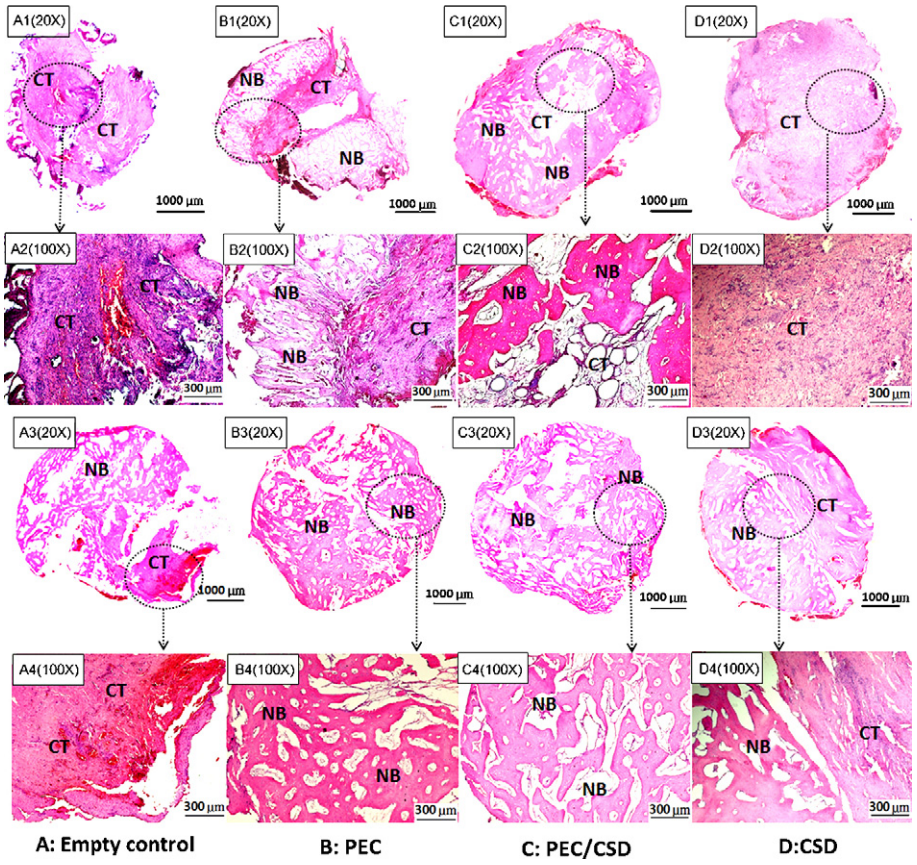

Fig. 6. Histological evaluation of defects without filler (empty control) (A), and defects filled with PEC (B), PEC/CSD (C), and CSD (granular) (D). Three weeks postoperatively: A1-2, B1-2, C1-2, and D1-2. Six weeks postoperatively: A3-4, B3-4, C3-4, and D3-4. Sections were stained with hematoxylin and eosin (magnifications, 20× and 100×).

4. Conclusions

In conclusion, a chitosan/PG based injectable PEC scaffold was successfully developed. This PEC system is promptly gelled under mild conditions without employing any toxic crosslinker, and it can take the shape of the cavity and fill irregular defects. At formulation of 8% chitosan and 3% PG + 2% CM-cellulose, the optimal result in vitro was obtained. The canine alveolar bone defects test suggests adding CSD to the PEC can promote osteogenic efficacy, however the exact mechanism of CSD effect for enhancing bone regeneration require further biochemical studies. In conclusion, these studies demonstrated that the PEC/CSD may have high potential for use in bone tissue engineering.

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