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A novel injectable chitosan/polyglutamate polyelectrolyte complex hydrogel with hydroxyapatite for soft-tissue augmentation

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ABSTRACT

This study demonstrated a chitosan (CS)/polyglutamate (PG) polyelectrolyte complex (PEC) hydrogel combined with spherical hydroxyapatite (HAp) particles as an injectable dermal filler for soft-tissue augmentation. The CS/PG PEC hydrogel with oppositely charged ionic cross-linking, a high gel content, and low degradation rate was introduced as a carrier to achieve high shape and volume stability. An MTT assay indicated that the CS/PG PEC had satisfactory cell biocompatibility. This PEC/HAp hydrogel showed good structural integrity in a PBS solution for up to 60 days. Clinical manageability was indexed by an injection force measurement through sterile 27-gauge needles using a texture analyzer. In an animal study, 0.2 mL of the PEC and PEC/hydroxyapatite (HAp) were implanted within the dorsal dermis of a swine ear. Injected tissue areas were biopsied 2 weeks, and 2 and 6 months after the injection. According to the histomorphometric results, the PEC and PEC/HAp groups showed percentages of retention of the maximum height of the cross-section of about 44% and 73% at 6 months. New collagen was observed in the central position indicating a possible collagenesis effect. These results suggest that this PEC/HAp system can be used as an alternative for soft-tissue augmentation.

1. Introduction

Soft-tissue augmentation is a nonsurgical procedure for injecting a substance in dermal or subcutaneous tissues for esthetics or supporting purposes with the advantages of a low risk of complications and a short recovery time. Based on the criteria of correction longevity, dermal fillers can be classified as temporary, semi-permanent, and permanent. Unlike permanent filler materials such as silicone which may eventually induce patients to develop granulomas (Lemperle, Morhenn, & Charrier, 2003), temporary dermal fillers such as collagen and hyaluronic acid generally elicit low immune responses but have short duration periods of 3–6 months (Broder & Cohen, 2006). On the other hand, semi-permanent particle filler systems such as hydroxyapatite (HAp) (Radiesse®, BioForm Medical, San Mateo, CA), β tricalcium phosphate (β-TCP) (Atléan®), and poly-L-lactic acid (PLA) (Sculptra®) loaded in carriers reveal longevity and collagen stimulatory properties (Johl & Burgett, 2006; Thioly-Bensoussan, 2008).

Radiesse® is a commercial product composed of a suspension of 30% HAp particles in the 70% carboxymethyl cellulose (CMC) carrier system. The safety profile of HAp, an inorganic constituent of teeth and bone, has been extensively evaluated (Havlik, 2002). After injection, the CMC carrier gradually undergoes degradation and macrophage phagocytosis over a period of 6–8 weeks (Flaharty, 2004). Finally, only HAp particles were left to provide mechanical volume support; thus additional treatment is usually planned for moderate or deep wrinkles after 6 weeks (Redbord, Busso, & Hanke, 2011; Thioly-Bensoussan, 2008).

Polyelectrolyte complexes (PEC), mixtures of two polyelectrolytes with opposite charges (cationic and anionic) and electrostatic interactions (Li et al., 2007), received considerable attention in the past several decades. PEC hydrogels prepared from natural polymers, such as polysaccharides and polypeptides, have additional advantages of being nontoxic and bioabsorbable (Cascone, Sim, & Downes, 1995). PEC cross-linked compositions are essential for the resulting gel properties for use in biomedical application (Dai et al., 2007; Wu et al., 2011).

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Chitosan (CS) is a natural cationic polysaccharide constituted of N-glucosamine and N-acetyl-glucosamine units (Muzzarelli, 2009, 2011). The biodegradable polyglutamate (PG) is a natural anionic polypeptide produced by *Bacillus subtilis* (Richard & Margaritis, 2001). Due to the particular physicochemical and mechanical properties of CS/PG-based PEC hydrogels, they have been proposed for wound dressing (Tsao et al., 2011), bone scaffolds (Wu et al., 2011), and skin substitutes (Berger et al., 2004). However, no report has so far used CS/PG-based PEC hydrogels combined with HAp as the dermal filler.

The aim of this study was to develop a PEC hydrogel formulation with dermal filler carrier properties. We examined the hydrogel in vitro (gel content, water uptake, degradation, injectability, structural integrity properties, and biocompatibility); moreover, HAp particles were added to the PEC hydrogel to provide support and longevity properties. The implanted materials were characterized in vivo using a swine soft-tissue model (histological and histomorphometric evaluation).

2. Materials and methods

2.1. Materials

CS (MW 200,000) with a degree of deacetylation of approximately 80% was obtained from Tokyo Chemical Industry (TCI, Tokyo, Japan). PG (MW 1000–10,000) was purchased from Vedan (Taichung, Taiwan). Phosphate-buffered saline (PBS), high-viscosity CMC, sodium hydroxide, potassium bromide, glycerin, and MTT were supplied by Sigma (St. Louis, MO, USA). Trypsin–EDTA, Dulbecco's modified Eagle medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco BRL Life Technology (Grand Island, NY, USA). Spherical HAp particles were prepared in our laboratory by a spraying, drying, and sintering method.

2.2. Preparation of specimens (PEC)

CS powder was dissolved in 1% acetic acid to prepare a CS solution at a concentration of 3 wt%. Different amounts of PG powder were added and well-dispersed in a previously prepared CS solution to make CS/PG PEC hydrogels at respective concentrations of 1–6 wt%. The pH value of the PEC hydrogels was adjusted to 6.8 by adding a 10 N NaOH aqueous solution and agitating the mixture. Finally, PEC hydrogels would put in room temperature for 24 h.

2.3. Characterization of PEC hydrogels

2.3.1. Gel content

PEC hydrogels were injected into a 12-well culture plate and dried in a freeze-drier (FD-122S-3P, Kingmech, Taipei, Taiwan) for 24 h. Specimens were lyophilized and weighed. Acetic acid (1%; the solvent for CS and PG) was used to extract non-cross-linked material from the specimens. The extraction bath ratio for acetic acid to PEC was 50:1. 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 solvent for 24 h, washed with double-distilled water (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.

2.3.2. Equilibrium water uptake

To measure the water uptake of PEC specimens, samples were dried using a freeze-drier. The dry weight (W_d) was immediately measured, and then specimens were immersed in PBS at 37 °C for 24 h, and weighed to obtain the wet weight (W_w). The swelling property of the matrices was calculated using the following equation (Jin et al., 2009):

$$\text{Water uptake}\,(\%) = \frac{W_w - W_d}{W_d} \times 100.$$

2.3.3. In vitro degradation test

To prepare the CMC solution, 3 g of CMC powder and 15 g of glycerin were dissolved in 52 g DDW. In accordance with ISO10993-9 guidelines, lyophilized specimens, including the PEC and CMC groups, were incubated in 50 mL of PBS at 37 °C with agitation at 50 rpm. After 1, 2, 4, 12, 26, 32, and 48 weeks of incubation, samples were removed from the PBS, rinsed with DDW, and lyophilized. The amount of degradation was indexed as the weight retention calculated by following equation:

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

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

2.4. Characterization of PEC hydrogels containing HAp particles

2.4.1. Structural integrity test

The PEC, PEC/HAp, and CMC/HAp groups were compared using an in vitro structural integrity test. The final concentration of HAp was 30 wt% in the PEC/HAp and CMC/HAp groups. Three groups were poured into a syringe and immediately injected into a PBS solution at room temperature, and then each group was photographed at 5 min (0 day), 1, 7, and 60 days.

2.4.2. In vitro injectability test

To conduct the injectability test, four groups (PEC, CMC, PEC/HAp, and CMC/HAp) were individually loaded into a 1-mL syringe with a sterile needle (27-gauge, 0.5 in.). The maximum injection force (F(max)) and dynamic glide force were recorded using a texture analyzer (TA Instruments, Wilmington, DE, USA) with syringe testing rig equipment at a crosshead speed of 15 mm/min.

2.5. In vitro cell compatibility

In accordance with ISO10993-5 guidelines, the cell compatibility of the PEC was evaluated on the NIH3T3 cell (fibroblast) 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. The 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, cellular responses were assessed by optical microscopy and the MTT assay. The absorbance, A_{570}, of MTT in each well was immediately recorded at 570 nm using ultraviolet (UV)/vis absorbance spectroscopy (Bio-Tek Instruments, Winooski, VT, USA). The cell compatibility was estimated using the following equation:

$$\text{Cell viability}\,(\%) = \frac{A_{570}\ \text{of the test material}}{A_{570}\ \text{of the control}} \times 100.$$

In the cell-attachment test, the PEC was sterilized in 75% ethanol for 24 h and washed with PBS. The 5000 NIH3T3 cells were seeded

on a PEC hydrogel and incubated at 37 °C in an incubator with a 5% CO_2 atmosphere for 72 h. Cell-containing specimens were fixed with a 2 wt% glutaraldehyde solution followed by treatment with 1 wt% OsO_4 (post-fixed). Specimens were dehydrated in a graded ethanol series (30, 50, 70, 80, 90, and 100 wt%), and then subjected to liquid CO_2 critical-point drying. Finally, cells containing the PEC were gold-coated and examined by scanning electron microscopy (SEM) (Hitachi S2400, Tokyo, Japan) under an accelerating voltage of 15–20 kV.

2.6. In vivo soft-issue augmentation test

The animal study was performed in strict accordance with protocols approved by the Animal Care Committee of Taipei Medical University (LAC-99-0093). Six Lanyu swines with a mean age of 3 months were supplied by the Taitung Animal Propagation Station (Taitung, Taiwan). Using a 27-gauge needle, 0.2 mL of filler materials was injected in the dorsal dermis of both ears of each swine, while under general anesthesia. PEC, PEC/HAp, and CMC/HAp fillers were injected into the respective experimental groups; the injection resulted in the appearance of a dome-shaped augmentation that could be easily palpated. The locations of the injection sites and type of material injected were marked on a transparent plastic sheet placed over the ear for later identification. Specimens were harvested after 2 weeks, and 2 and 6 months, and the resultant soft-tissue augmentation was examined by histological and histomorphometric methods.

Each implanted site was harvested at 2 weeks, and 2 and 6 months after the injection. Biopsies obtained from each site were cut to a specific area (2 cm × 2 cm). Harvested specimens were fixed in 10% buffered formalin for 1 week, and were cut perpendicular to the skin surface with a surgical blade at a thickness of 5 mm (Pitaru et al., 2007). Finally, specimens were made into paraffin sections and stained with Masson's trichrome stain to investigate the collagen fiber density in the dermis. Each specimen was examined for the shape of the injected materials, preservation of its initial shape over time, the host response at the implant site, and microscopic degradation of the implanted materials. Stained sections were observed under optical microscopy (Olympus EX51, Tokyo, Japan). A histomorphometric method was used to quantify the amount of soft tissue within the augmentation area. The maximum augmentation height of a cross-section was determined and quantified using Image J analysis software (National Institutes of Health, Bethesda, MD, USA).

2.7. Statistical analysis

The mean and standard deviation (SD) of the maximum height of a cross-section for each group of filler materials was calculated for various implantation time periods. Data were compared using a one-way analysis of variance (ANOVA) to evaluate the statistical significance of the measured data. Post hoc Tukey's test was used to compare the significance of deviations in the measured data of each group. In all cases, differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Characterization of the PEC hydrogels

3.1.1. Gel content and equilibrium water uptake

The gel content is a basic parameter to calibrate gel formation due to cross-linking because not all of the macromonomers eventually join the gel network. Fig. 1 displays the gel content for various PEC formulations. Each sample was determined by measuring its insoluble part after extraction in solvent. The highest and lowest

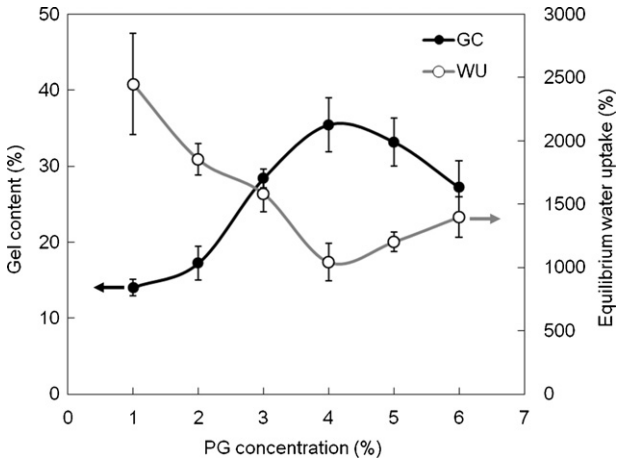

Fig. 1. Gel content (GC) at 25 °C in an acid solution (●) and equilibrium water uptake (WU) at 37 °C in PBS for 24 h (○) of PEC hydrogel formed by mixing different concentrations of PG with 3% chitosan (CS) ($n = 4$).

gel contents were 35.46% and 14.08% for samples with 4% and 1% PG, respectively. The swelling property is an important parameter to characterize cross-linked structures. The ability to hold sufficient amounts of water inside the network structures is an important parameter to characterize the swelling properties of hydrogels (Jin et al., 2009). Equilibrium water uptake values of the designed specimens were within 1043–2448% as shown in Fig. 1. According to the above results, the PEC hydrogel formulation with the high gel content had a lower water uptake value (1043%). Therefore, based on the above results, a composition of 4% PG and 3% CS may have been nearest to the stoichiometric formulation and was chosen for the following experiments.

The swelling property inversely matched the gel content of the hydrogels (Sun, Cao, Su, & Tan, 2009). Hydrogels with the higher gel content had a lower swelling ratio which was largely restricted by the cross-linking density. The gel content of the PEC was associated with the swelling behavior. The higher gel content resulted in a higher cross-linked density, which confines the macromolecules to a limited spatial volume; so the water uptake value is largely restricted. Moreover it caused lower sample volume expansion and avoids tissue oppression in bodily fluids (Peter et al., 2010; Wu, Ji, Chang, Yang, & Lee, 2012).

The gel content and swelling behavior of the hydrogels were affected by different molecular weights when treated with the same cross-linking method (Tranquilan-Aranilla, Yoshii, Dela Rosa, & Makuuchi, 1999). Hyaluronic acid with methacrylic anhydride to modify the hydrogel is one of the simplest and most widely used reactions (Burdick & Prestwich, 2011). Swelling ratios of hyaluronic acid-cross-linked hydrogels range 8–42 (Burdick, Chung, Jia, Randolph, & Langer, 2005). In a previous study, a stoichiometric formulation of a PEC hydrogel also had a long degradation property (Tsao et al., 2011; Wu et al., 2011).

3.1.2. In vitro degradation test

In accordance with ISO10993-9 guidelines, weight loss of the PEC in PBS at 37 °C was measured over time. Fig. 2 indicates rapid weight loss of the PEC in the first week (51.1% weight loss); afterwards, the degradation rate decreased. During the period of 1–48 weeks, the shape of the PEC was almost a completely intact morphology; thus the weight retention of the designed PEC cross-linked structure was 22.4% at 26 weeks and even 15.4% at 48 weeks.

A modified CS-alginate gel system showed degradation of 38.6% within 28 days (Jin et al., 2009). Wu et al. (2011) reported a CS-based PEC hydrogel, which degraded by about 27.7% and 47.4% within 1 and 26 weeks. According to Tsao et al.'s report, a CS/PG PEC

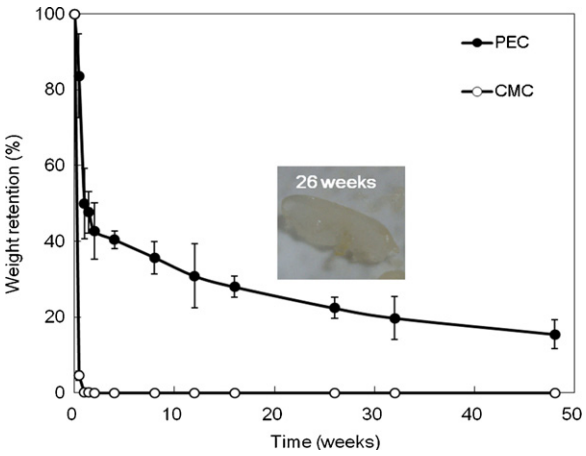

Fig. 2. Degradation profile of CMC and the PEC hydrogel at 48 weeks in PBS at 37 °C ($n = 3$).

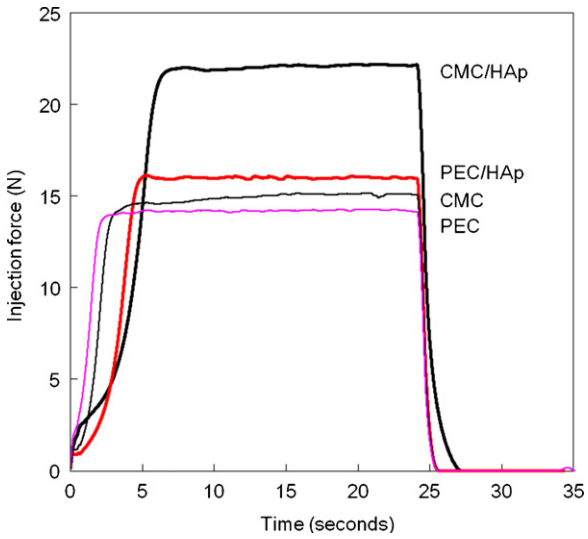

Fig. 4. Injection properties with 27-gauge needle of the PEC hydrogel, CMC, PEC/HAp, and CMC/HAp.

hydrogel showed degradation of 21% within 28 days. In this study, the degradation of the PEC hydrogel was about 59.6% at 28 days and 76.6% at 26 weeks. This system could be maintained for more than 26 weeks.

3.2. Characterization of PEC hydrogels containing HAp particles

3.2.1. Structural integrity test

Fig. 3 shows the structural integrity of the PEC hydrogel, PEC/HAp and CMC/HAp systems which were injected into a PBS solution for 5 min (day 0), 1, 7, and 60 days. The extent of decay of the CMC/HAp group increased with prolongation of the immersion time. The CMC/HAp hydrogel began to disintegrate after immersion in water for 1 day and had completely lost its volume support after 7 days. The PEC hydrogel was transparent and showed a swollen morphology due to physical cross-linking (day 0). The PEC hydrogel had an integrated structure with time and showed only a small

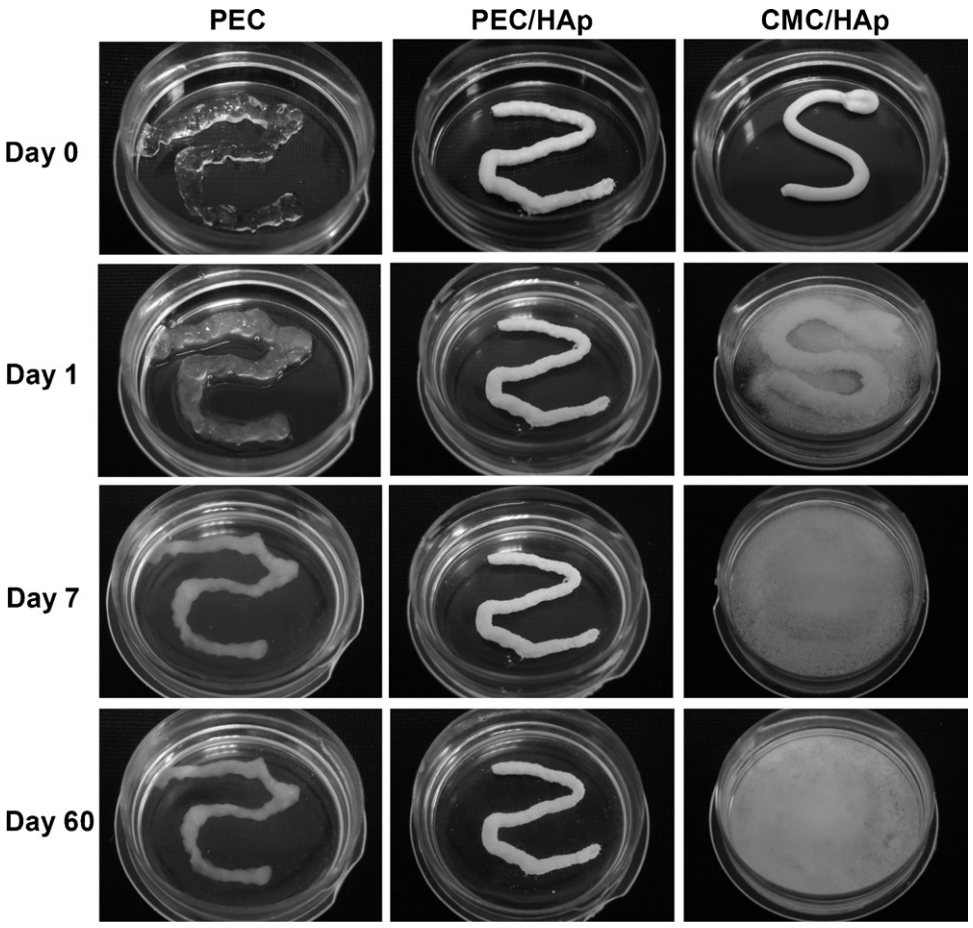

Fig. 3. Structural integrity properties of the PEC hydrogel, PEC/HAp, and CMC/HAp at 25 °C in PBS for different times.

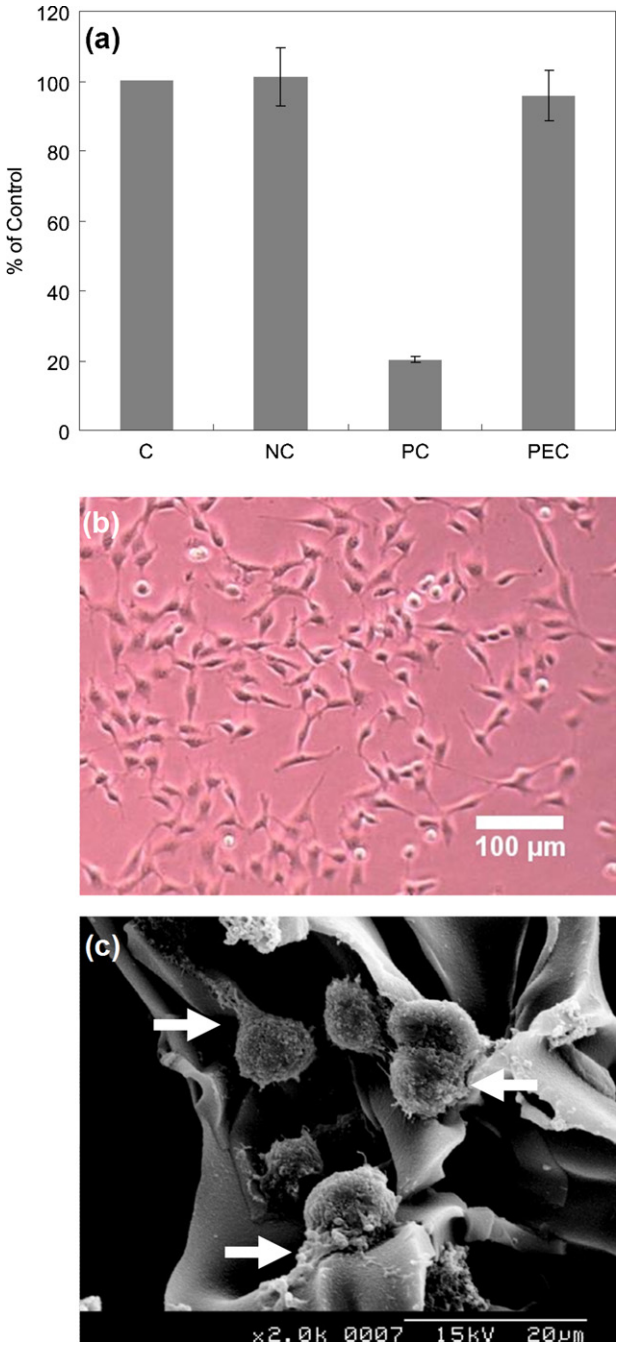

Fig. 5. (a) MTT reduction by NIH3T3 fibroblast cells exposed to the PEC extract compared to MTT reduction in the control (C) (cells with medium alone), negative control (NC) (cells with high-density polyethylene), and positive control (PC) (cells with 0.5% DMSO) for 24 h. (b) Optical photomicrograph (100×) of NIH3T3 fibroblast culture in PEC hydrogel extract medium. (c) Scanning electron micrographs showing the growth of NIH3T3 cells on the PEC hydrogel for 3 days (magnification, 2000×). The cell density was 10^6 cells/mL.

reduction in volume after day 7. The PEC/HAp hydrogel remained almost stable in its initial shape, and no obvious decay or disintegration was observed at 60 days. However, the supporting volume ability and structural integrity of the PEC/HAp hydrogel were stronger than those of the CMC/HAp hydrogel.

Structural integrity is discussed in terms of preventing washout of injectable bone cement supporting the original shape and dimensions (Lin et al., 2010). When the PEC hydrogel carrier was introduced to the filler system, a network that spread all

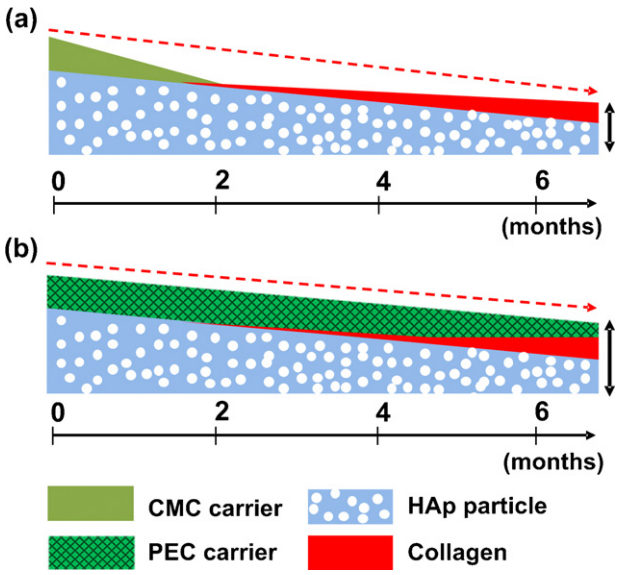

Fig. 6. Schematic illustration of time-dependence degradation and collagenesis model for (a) CMC/HAp and (b) PEC/HAp dermal fillers.

over the HAp particles was formed, resulting in good maintenance of its shape even after exposure to PBS. The high viscosity and polyelectrolyte reaction between the PEC hydrogel and HAp helped the filler system retain its original position and shape. As a result, the ability of the dermal filler to maintain its structural integrity was dramatically enhanced with the addition of the PEC hydrogel.

3.2.2. *Injection properties test*

An ideal injection property is to be able to deliver the dermal filler through a large-gauge needle, so it must possess low viscosity at high shear (Falcone, Doerfler, & Berg, 2007). The ease of injectability (low injection force) of dermal fillers is important to clinicians (e.g., hand fatigue) and comfort for patients to improve their treatment experience and satisfaction (Lorenc, Nir, & Azachi, 2010). The injection force is the minimum force applied to the syringe to achieve injection (Tezel & Fredrickson, 2008).

Fig. 4 plots the force necessary to expel the samples through a 27-gauge needle from a 1-mL syringe. For comparison, respective injection forces required for PEC, CMC, PEC/HAp, and CMC/HAp were about 14, 15, 16, and 22 N. The PEC hydrogel groups (PEC and PEC/HAp) required less force than the CMC solution groups (CMC and CMC/HAp). The injection force of a commercial CMC/HAp dermal filler (Radiesse®) through a 27-gauge needle with a 1.3-mL syringe was nearly 6.0 lbf (about 27 N) (Busso & Voigts, 2008). Cilurzo et al.'s study reported a range of injection forces of 125–160 mPa (45–57.6 N) with some difficulty, and values of <125 mPa (45 N) had smooth properties. Thus, the PEC as a dermal filler offers several advantages over the preformed augmentation approach in terms of ease of injection and minimizing the invasiveness of surgery.

3.3. *In vitro cell compatibility and cell attachment*

Cell compatibility is an important characteristic of a material intended for biomedical applications. Cell compatibility was evaluated in NIH3T3 cells (fibroblast) by the MTT assay according to ISO10993-5 guidelines. Results are shown in Fig. 5a, and the PEC, control, and negative control groups showed no statistically significant differences, which reveals that the PEC hydrogel had little

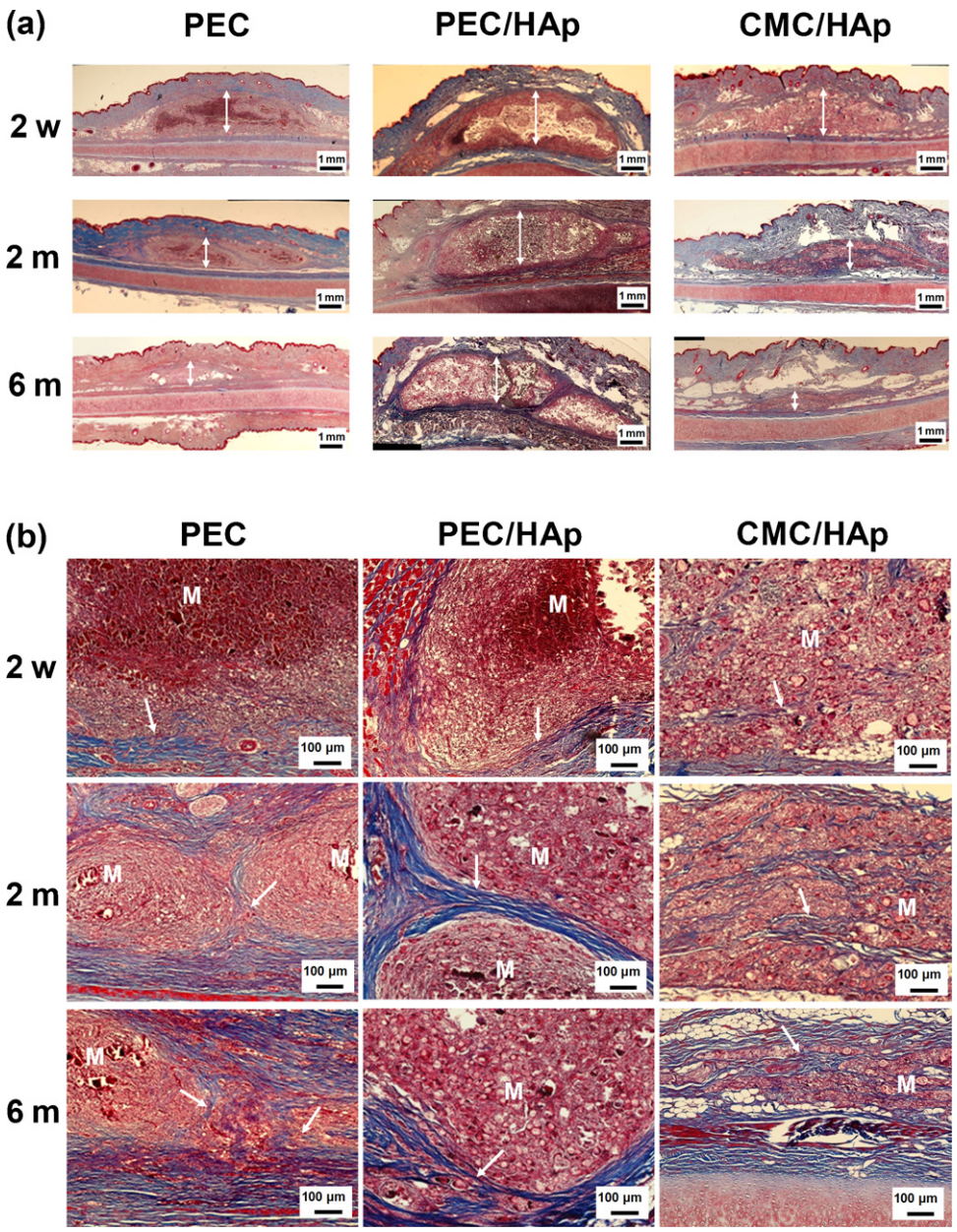

Fig. 7. (a) Dome-shaped appearance of the implanted PEC hydrogel, PEC/HAp, and CMC/HAp specimens examined throughout the course of the study. The white double-pointed arrows indicate sites of the maximum height measured in each section. (b) Epidermal aspect of the implants after different implantation times illustrating cellularity new collagen bundles (arrows) which were deposited in between the matrix of the implant materials (M) (magnification, 20× and 200×). (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

cytotoxicity (95.9%). Moreover, values of the positive and negative controls were 20.3% and 101.2%, respectively, which proves that the tests were done correctly. Cells cultured with extracted medium of the PEC are shown in Fig. 5b; the extraction induced no cell morphological changes of shrinkage or lysis. SEM photographs (Fig. 5c) show that NIH3T3 cells had adhered to the material surface, and the pseudopodia were well extended on it. Thus, the designed PEC hydrogel displayed satisfactory biocompatibility and cell affinity. Moreover, there was little toxicity evident in the cell-compatibility test. In comparison, hydrogels using a chemical cross-linking agent may be associated with cytotoxicity (Huang-Lee, Cheung, & Nimni, 1990), and physical ionic cross-linking displayed much fewer biocompatibility problems. Our PEC hydrogel may be able to provide better stability and more-satisfactory biocompatibility than chemical cross-linking methods.

3.4. In vivo histological and histomorphometric evaluations of soft-tissue augmentation

Three injected materials (PEC, PEC/HAp, and CMC/HAp) were located in the dermis between the epidermis and ear cartilage and were harvested at 2 weeks, and 2 and 6 months postoperatively. The material degradation and tissue collagenesis response with time of two dermal fillers (CMC/HAp and PEC/HAp) in the animal model are shown in Fig. 6.

Host tissue responses to an implanted material can be evaluated using histologic methods. Histological sections of skin were subjected to Masson's trichrome staining to visualize extracellular matrix (ECM) components and morphological changes. The soft-tissue augmentation efficacy was evaluated by histological and histomorphometric methods. Fig. 7 shows the staining results of

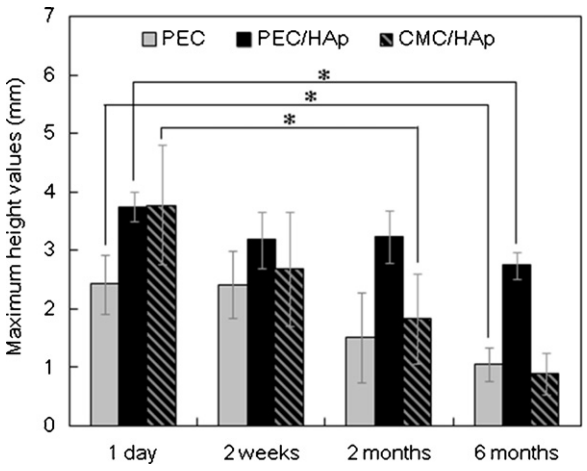

Fig. 8. Mean and standard deviation of the maximum height throughout the experiment of the three implant types: PEC hydrogel, PEC/HAp, and CMC/HAp. *Significant difference in the same group at different times ($p < 0.05$).

collagen fiber (blue) in the PEC, PEC/HAp, and CMC/HAp groups. At all time points examined, the host tissue responses in the three groups were very good. There was no evidence of any acute or inflammatory reaction to these materials. These findings demonstrate their high biocompatibility.

Histological pictures of the support efficacy of the materials are given in Fig. 7a. In the PEC group, a cross-section of the injected hydrogel was dome-shaped, the base of which was located in the deep part of the dermis. Unresorbed hydrogel still remained in the central position at 2 and even 6 months. That hydrogel dome appearance to have been preserved during the experimental period. The PEC hydrogel had well maintained its maximum height at 6 months and slowly decreased over time. At 2 weeks, the appearance of the cross-section of the CMC/HAp group was similar to the PEC/HAp group in exhibiting a dome-like shape. The thickness of the CMC/HAp group dropped rapidly over time; it had a thin plate-like appearance at 6 months. In that system, the CMC carrier was mostly degraded, while the hydroxyapatite filler remained at 2 months. The PEC/HAp group maintained its maximum height efficacy better than did the CMC/HAp group.

New collagen (blue color) was produced by fibroblast cells that surrounded the injected materials (deep red color), and it slowly grew into the injected central position at 2 and 6 months (Fig. 7b). At 2 and 6 months, fibroblast cells had colonized areas of the CMC/HAp material and secreted new collagen that was deposited within the implanted HAp particle cavities where the CMC carrier had degraded. On the other hand at 2 and 6 months, new collagen was seen around the PEC/HAp material and had slowly surrounded the unresorbed materials in the central position. A marked increase in collagen was observed in the CMC carrier group which rapidly degraded. However, the dermal thickness was not more than those of the PEC hydrogel carrier groups and demonstrated integration of the implant within the dermal tissue and collagenesis.

Results of the histomorphometric analysis of the implant maximum height are shown in Fig. 8. We tried to quantify the change in the shape of each group during the experimental period. The maximum heights of the PEC/HAp material were similar at 2 months (3.24 ± 0.45) and 1 day (3.75 ± 0.26) and did not statistically differ ($p > 0.05$). The maximum height ratios dropped by 14% at 2 months and 27% at 6 months compared to the height on day 1. In contrast, the maximum height of the CMC/HAp material was statistically significantly ($p < 0.05$) 51% lower at 2 months (1.83 ± 0.78) than on day 1 (3.78 ± 1.02). The PEC hydrogel group was approximately 56% lower at 6 months (1.05 ± 0.28) than its maximum height at 2 weeks (2.42 ± 0.58), and the difference was statistically significant because of the high variation observed at 6 months ($p < 0.05$). There was no significant different ($p > 0.05$) between day 1 (2.42 ± 0.50) and 2 months (1.52 ± 0.77).

In vivo efficacy of the PEC hydrogel was estimated using a swine ear dermis model due to the ease of evaluating the efficacy of the dermal fillers (Hemmrich et al., 2008; Pitaru et al., 2007). The metabolic rate and dermal structure of the swine were described as perhaps being the most similar to humans (Boza, Cunha, de Andrade, & Palma Kuhl, 2011; Pearce, Janardhan, Caldwell, & Singh, 2007). One drawback of this animal model is the fact that in the swine model described in this study, the filler materials were injected into young animals, whereas in humans, they are intended for use in an aging population. Thus, the tissue response and aging processes might differ between the two species, and linear extrapolation from animals to humans is not necessarily valid. However, we conducted a histological evaluation of soft-tissue augmentation in an in vivo swine ear model.

In this animal study, the PEC system provided a hydrogel that retained its shape and three-dimensional structure after implantation for up to 6 months. Whether the carrier with cross-linking structures or not affected the long-term efficacy and shape retention of dermal fillers. In the collagen dermal filler study, the 6-month maximum height was 55% lower than that at 1 month with non-cross-linked collagen, but cross-linked collagen was only reduced by 27% even at 24 months (Pitaru et al., 2007). Ma et al.'s animal research proved that the collagen/CS hydrogel scaffold can induce fibroblast cell infiltration from surrounding tissues. The long-term PEC hydrogel system might prove to be an appropriate scaffold for fibroblasts to synthesize collagen.

The CMC carrier system at the injection site resulted in a very rapid reduction in the maximum height of the dome-like structure and consequently a change in the three-dimensional shape of the implant over time. England et al. (2005) demonstrated fibroblast replacement of the aqueous CMC carrier, and their results were similar to those of the CMC/HAp group in our study. Their findings showed collagen surrounding the HAp particles 2 months after the injection, but collagen synthesis was as fast as the degradation rate of the CMC gel carrier to support the soft tissue (Marmur, Phelps, & Goldberg, 2004).

According to the histomorphometric results (Fig. 8), we also compared the efficacy of the PEC hydrogel and PEC/HAp. HAp particles indeed enhanced the augmentation and support properties of the PEC hydrogel, especially in the early stage. Because of their good support efficacies, dermal fillers have replaced some conventional surgery for facial rejuvenation (Jacovella, 2008; Vedamurthy, 2004), vocal cord adjustment (Ford & Bless, 1986), stress urinary incontinence (Mayer, Lightfoot, & Jung, 2001), and vesicoureteral reflux (Galus, Antiszko, & Wlodarski, 2006). The PEC/HAp injectable dermal filler system may be able to meet all of the above requirements.

4. Conclusions

In conclusion, a CS/PG-based PEC hydrogel with HAp was developed for soft-tissue augmentation. This dense PEC hydrogel system promptly gels under mild conditions without employing any toxic cross-linker. The most suitable PEC hydrogel system had a high gel content (35.46%), low water uptake value (1044%), and slow degradation rate. The cytotoxicity and cell attachment tests of the PEC hydrogel showed satisfactory cell compatibility. The PEC/HAp showed good structural integrity properties and injectability (16 N) compared to CMC/HAp. Moreover, in vivo studies of the degradation of PEC/HAp indicated that it had long-term stability, and new collagen was slowly synthesized as a result. The PEC hydrogel can provide the HAp filler that promotes support efficacy (volume and

height). For this reason, the novel CS/PG PEC hydrogel with HAp may be a good potential material for soft-tissue augmentation.

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