JOURNAL OF Applied Polymer SCIENCE

The Effect of BMP-2 and VEGF Loading of Gelatin-Pectin-BCP Scaffolds to Enhance Osteoblast Proliferation

Jhaleh Amirian,[1] Nguyen Thuy Ba Linh,[1,2] Young Ki Min,[3] Byong-Taek Lee[1,2]

[1]Department of Regenerative Medicine, College of Medicine, Soonchunhyang University 366-1, Ssangyong-Dong, Cheonan-City, ChungCheongNam-Do 330-090, Republic of Korea

[2]Department of Regenerative Medicine, Institute of Tissue Regeneration, Soonchunhyang University 366-1, Ssangyong-Dong, Cheonan-City, ChungCheongNam-Do 330-090, Republic of Korea

[3]Department of Physiology, College of Medicine, Soonchunhyang University 366-1, Ssangyong-dong, Cheonan-City, ChungCheongNam-Do 330-090, Republic of Korea

Correspondence to: B.-T. Lee (E-mail: lbt@sch.ac.kr)

ABSTRACT: A composite scaffold of gelatine (Gel)-pectin (Pec)-biphasic calcium phosphate (BCP) was successfully fabricated. Growth factors such as bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF) were loaded into the Gel-Pec-BCP hydrogel scaffolds by freeze-drying. The surface morphology was investigated by scanning electron microscopy, and BCP dispersion in the hydrogel scaffolds was measured by energy dispersive and X-ray diffraction spectroscopy. The results obtained from Fourier transform infrared spectroscopy and quantitative measurements showed successfully loading of BMP-2 and VEGF into the Gel-Pec-BCP hydrogel scaffolds. In addition MC3T3-E1 preosteoblasts were cultivated on the three types of scaffolds to investigate the effects of BMP-2 and VEGF on cell viability and proliferation. The Gel-Pec-BCP scaffolds loaded with VEGF and BMP-2 demonstrated more cell spreading and proliferation compared to those of the Gel-Pec-BCP scaffolds. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41241.

KEYWORDS: biocompatibility; biomaterials; porous materials

Received 25 March 2014; accepted 30 June 2014
DOI: 10.1002/app.41241

INTRODUCTION

Bone tissue engineering materials have gain more attention in recent years because of specific advantages to the repair and regeneration of damaged bone tissue over allografts and autografts, without causing injuries such as pain and donor site morbidity, and without other risks such as vessel injury during the harvesting of bone from the donor.[1–4] In addition, biomaterials have been developed due to their excellent biocompatibilities for pharmaceuticals, drug delivery, and tissue engineering.[1,2,5–8] Consequently, the preparation of a scaffold with optimal architecture is the most important factor for bone tissue regeneration. In the case of bone tissue engineering, a high porosity (~90%), interconnected network with appropriate pore size is an essential feature for the design.[1,2,9–11]

Additionally, scaffolds have been used for delivering bioactive factors, such as bone morphogenetic proteins (BMP-2), transforming growth factors (TGF), and vascular endothelial growth factors (VEGF), which are an important group of bioactive molecules, which play important roles in bone regeneration. High osteoinductive potential can be seen in some BMPs and TGFs.[2,12–17]

Apart from osteoinduction, angiogenesis is also necessary for bone regeneration, as the formation of new vessels helps blood to transport the nutrient supply to organs.[12,17–19] Several kinds of natural and synthetic polymer materials have been investigated, both for the purpose of drug delivery and tissue engineering, because of their biocompatibility, biodegradability properties, availability, and low cost. These include polymers such as gelatin (Gel),[20,21] collagen,[22] pectin,[23–27] oxidized alginate,[14] fibrin,[13] and hyaluronic acid (Hya)[22,28,29] poly(DL-lactide-*co*-glycolide) (PLGA)[15] and poly(L-lactic acid) (PLLA).

Gelatin, as water soluble protein fragment, is obtained with partial hydrolysis of collagen,[30,31] although previous studies reported that collagen itself shows good properties such as biocompatibility, noncytotoxicity, and an ability to support cellular growth.[31,32] However, usage of collagen has been limited because of certain properties such as poor dimensional satiability due to swelling *in vivo*, poor *in vivo* mechanical strength, low elasticity, and the possibility of an antigenic response that could cause tissue irritation due to the residual aldehydes crosslinking agents.[33] In addition, pure Type I collagen has a high cost.[31] This led to wide use

of gelatin instead of collagen, as it also has good properties such as cell adhesion, migration, differentiation, and proliferation in tissue engineering applications.[21,32] Moreover, use of Gelatin instead of collagen can reduce the cost of the materials for scaffold fabrication. By incorporating other polysaccharides such as pectin, a biocompatible system can be created, which is also suitable for drug delivery and tissue engineering.[23]

Pectin, as a natural carbohydrate, is composed of heteropolysaccharides from plant cell wall components.[25,34,35] Pectin has gained attention due to several special properties such as high water content, and the ability for homogenous immobilization of cells, gens, proteins, drugs, or growth factors. Furthermore, because of its ability to form a gel in the presence of divalent cations, pectin is an ideal carrier for drug delivery of bioactive agents.[24,25,34–36] On the other hand, bone regeneration was reported to be accelerated in the presence of ceramics, including synthetic calcium phosphate (BCP) and hydroxyl apatite (HAp), which makes them applicable for bone tissue engineering scaffolds.[37,38] As ceramics are fragile, the applications have been limited to use with polymers.[39,40]

Considering the properties discussed above, scaffolds made from a combination of gelatin-pectin-BCP may be ideal for bone tissue engineering applications.[41] In this study, we prepared three types of scaffolds, including freeze-dried Gel-Pec-BCP, BMP-2 loaded Gel-Pec-BCP, and VEGF loaded Gel-Pec-BCP scaffolds prepared using a freeze-drying method, and investigated the release and cell proliferation behavior of BMP-2 and VEGF in the Gel-Pec-BCP hydrogel systems.

EXPERIMENTAL

Materials

Gelatin (Gel, Sigma-Aldrich, St. Louis, MO), pectin from citrus (Pec; Sigma-aldrich, Denmark) and BCP were used as starting materials for fabricating the Gel-Pec-BCP composite scaffolds. BCP powder (particle size, 90–100 nm) was synthesized using a microwave-assisted process.[42]

Ethanol (Merck, Darmstadt, Germany), fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA (Gibco, Carlsbad, CA), phosphate buffered saline tablets (PBS) (Amresco, Seoul, Korea), and α-minimum essential medium (α-MEM) (Gibco) were also used. MC3T3-E1 cells (American Type Culture Collection; ATCC-CRL-2593, Manassas, VA) were derived from mouse preosteoblasts.

Preparation of Gel-Pec-BCP Scaffolds

Gel (5 wt %) was dissolved in distilled water at 30°C. An identical amount of pectin (5 wt %) was dissolved in water, and then added to the Gel solution and mixed for 3 h. BCP (5 wt %) powder was added to deionized water, and then to the premixed gelatin-pectin solution until it became a slurry. The slurry was then mixed with a magnetic stirrer for 3 h. After mixing, the 2 mL slurry was poured into a polyethylene mold with a diameter of 12 mm and a height of 2 cm.[41] It was first frozen at −20°C for 10 h to prepare the porous scaffolds, and then placed in a freeze-dryer for 2 days.

The porous scaffolds were crosslinked using EDC (*N*-(3-dimethylaminopropyl) *N*′-ethylcarbodimide hydrochloride, Sigma-Aldrich) and NHS (*N*-hydroxysuccinimide, Sigma-Aldrich) at a 5:2 molar ratio in 80% ethanol overnight at 4°C. This step was repeated for stabilization of the scaffolds to prevent degradation during cell culture. The scaffolds were washed five times with distilled water for 15 min to remove residual EDC, frozen at −20°C for 3 h, and then dried. The amount of material (percent) and crosslinking depended on the degradation, and were optimize in a previous study.[41]

Loading BMP-2 and VEGF

Recombinant human rhBMP-2 (R&D Systems, Minneapolis, MN) and rhVEGF (R&D Systems) were loaded into the Gel-Pec-BCP in the slurry stage at 1 μg/mL, and were stirred separately for 5 min.

Characterization

Material Properties. Scaffold morphology was characterized by scanning electron microscopy (SEM, JEOL, JSM-6701F, Tokyo, Japan) equipped with an energy dispersive spectrometer (EDS). SEM/EDS provides a chemical analysis of the field of view, or spot analyses of minute particles. A small part of the scaffold tested was placed on the SEM sample holder and sputter coated with platinum. An accelerating voltage of 10 kV was used to obtain SEM images. A mercury porosimeter (Poremaster TM, Quantachrome Instruments, Boynton Beach, FL) was used to analyze the porosity and pore size distribution of the scaffolds. Low pressure intrusion porosimetry was used to obtain the interconnected macro pore diameter and porosity, as well the width × length × height (5 × 6 × 6 mm) dimensions.

X-ray diffraction analysis (XRD) (D/MAX-250, Rigaku, Tokyo, Japan, $\lambda = 1.541$ nm, 40 kV) was used for the crystal analysis. XRD analysis at a scanning speed of 20 min^{-1}, and Fourier transform infrared spectroscopy (FTIR, C1000 Thermal Cycler) in the range of 4000–400^{-1} were used for characterization of the freeze-dried scaffolds.

Micro-CT Analysis. Scaffolds produce via freeze drying were analyzed by micro X-ray computed tomography (micro-CT) (Skyscan 1076, Skyscan, Belgium). Scaffolds were mounted on a holder and placed in the scanner, after which imaging was performed for 360° of rotation with an exposure time of 20 min. Image reconstruction was performed using NRecon (Skyscan), and analysis of the reconstructed images was performed using CTAn (Skyscan) to obtain porosity values, including percentage of total porosity, open porosity, and closed porosity. In addition, DataViewer was applied to show the 3D view of the scaffold in the X, Y, and Z vectors.

***In Vitro* BMP-2 and VEGF Release Test.** The levels of BMP-2 and VEGF loaded into the Gel-Pec-BCP hydrogels were determined by BMP-2 and VEGF enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). To evaluate the amount of BMP-2 and VEGF released from the Gel-Pec-BCP-BMP-2 and Gel-Pec-BCP-VEGF hydrogel scaffolds, the specimens were soaked in 1 mL PBS at 37°C. The supernatant was collected and replaced with fresh PBS at intervals of 1, 2, 3, 5, 7, 10, 12, 14, 16, 18, 21, and 28 days. The absorbance of BMP-2 and VEGF in the supernatant was determined with the BMP-2 and VEGF ELISA kits, according to the manufacturer's instructions, using

a microplate reader (ELISA EL 312, Biokinetics reader, Bio-Tek instruments, Winooski, VT) at a wavelength of 450 nm.

Cell Culture and Seeding. MC3T3-E1 preosteoblasts were purchased from the American Type Culture Collection (ATCC-CRL-2593, Manassas, VA) and cultured in α-MEM, 10% FBS 100 U/mL penicillin, and 100 U/mL streptomycin.

Cell Viability Assay. The cell viability of each Gel-Pec-BCP composite was determined using the MTT assay, with a standard testing protocol [ISO 10993-5:2009(E)]. Briefly, the specimens were placed in a 24-well culture plate. They were immersed in 70% ethanol for 15 min for sterilization, and then washed in PBS to eliminate the ethanol. One milliliter (about 1×10^4 cell/well) of MC3T3-E1 preosteoblast suspension was placed in each well. The cell-seeded scaffolds were maintained at 37°C under 5% CO_2 conditions for 1, 3, and 7 days. After incubation, MTT solution(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 100 mL of 5 mg/mL, Sigma-Aldrich) was added to the wells, and the plate was incubated at 37°C for 4 h. Finally, the solution was removed from the plate, 1000 μL of dimethylsulfoxide (DMSO, Samchun Pure Chemical Co, LTD, Korea), was added to each well, and the plate was incubated for 1 h to form purple formazan crystals. Absorbance of the solution was determined at a wavelength of 595 nm using an ELISA reader. Samples were tested in quadruplicate.

Cell Proliferation. An aliquot of 1×10^5 cells were seeded on scaffolds in 24-well plates. After 1, 3, and 7 days of culturing in a humidified 5% CO_2 incubator at 37°C, the scaffolds were washed with PBS three times and then fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature. After three washes with PBS, the cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 10 min. This procedure was followed by addition of 2.5% bovine serum albumin for 60 min as a blocking reagent. The cells were immunostained using fluorescein isothiocyanate (FITC) conjugated phalloidin (25 μg/mL Sigma) overnight at 4°C in the dark. Nuclei were stained with 1 μg/mL Hoechst 33342 (2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate; Invitrogen). The scaffolds were visualized under a confocal fluorescent microscope (Olympus, FV10i-W, Tokyo, Japan), and the images were analyzed using the FV10i-ASW 2.0 Viewer software.

Cell Morphology. An aliquot of 1×10^5 cells were seeded on scaffolds in 24 well plates. After 1, 3, and 7-day incubation periods, the cell morphology was assessed under SEM to qualitatively analyze the MC3T3-E1 cell attachment and spreading on the three different types of scaffolds. In preparation for SEM, cultured samples were collected, rinsed with phosphate buffered saline (PBS), and subsequently fixed with 2% glutaraldehyde for 15 min. They were then treated with a gradation of ethanol, followed by treatment with 1,1,1,3,3,3-hexamethyldisilizane (HDMS) (Daejung, South Korea) for 10 min, and vacuum drying. Finally, the scaffolds were sputter-coated with platinum and viewed by scanning electron microscopy (SEM, JEOL, JSM-6701F, Tokyo, Japan).

RESULTS AND DISCUSSION

Characterization of Microstructure and Material Properties of the Gel-Pec-BCP Composite

The SEM micrographs showed low magnification of the surface and cross-sections of the scaffolds, confirming the highly interconnected pores of the Gel-Pec-BCP composite scaffold [Figure 1(a,b)]. At high magnification, the BCP particles embedded in the hydrogel could be observed [Figure 1(c)], with interconnection of the pores (100–300 μm) on the surface and in the interior of the hydrogel system [Figure 1(a, b, d, e1, e2, and e3)]. The interconnectivity of the scaffold can also be seen in the micro-CT image [Figure (e1, e2, and e3)], which also allowed measurement of the total percent of porosity and open porosity, which were around 91.40% and 91.37%, respectively [Figure 1(f)]. These data matched well with the SEM micrographs and mercury porosimetry, for interconnectivity and pore size distribution. The pore size obtained was suitable for bone regeneration.[43]

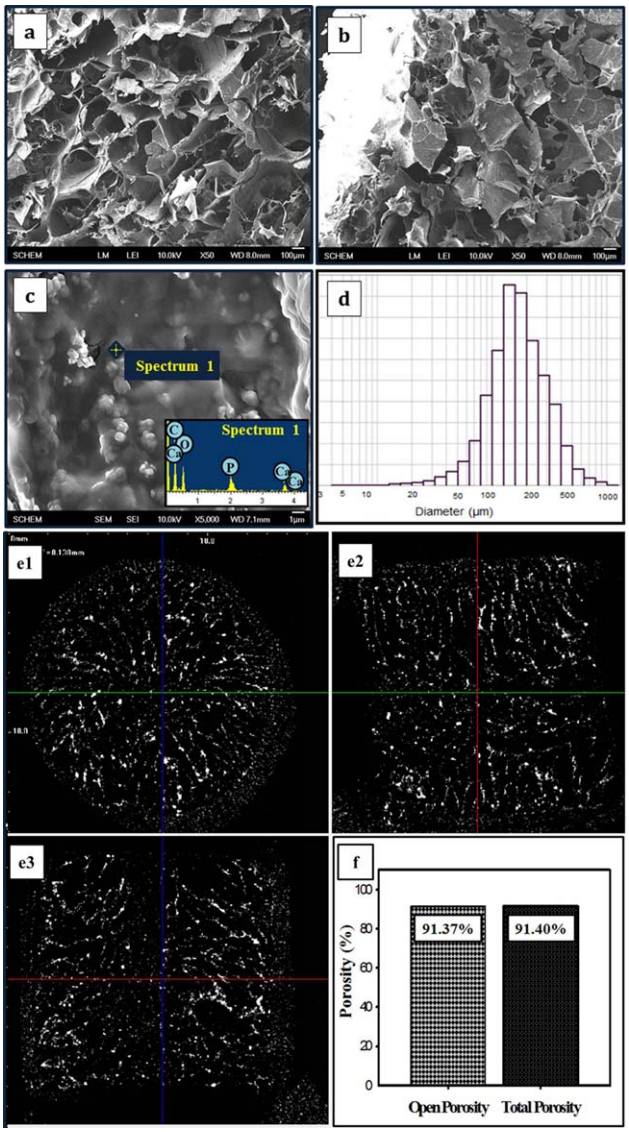

Figure 1. Scanning electron micrographs of: (a) the surface, (b) a cross-section of the Gel-Pec-BCP scaffold, (c) a high resolution image of the Gel-Pec-BCP with EDS profile (inset), (d) pore size of the Gel-Pec-BCP scaffold, (e) three-dimensions of the micro-CT image (e1–e3), and (f) value of porosity percent. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

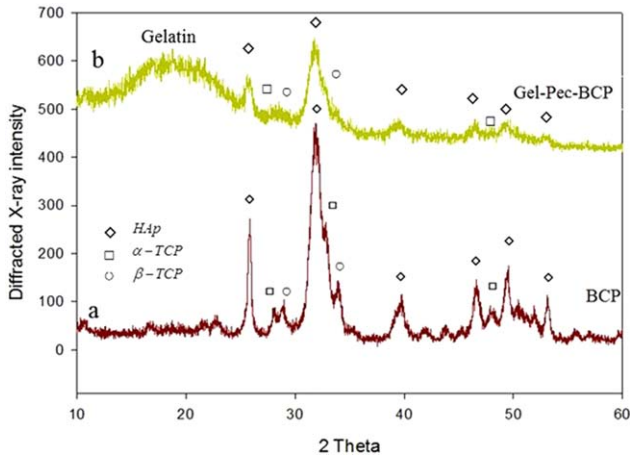

Figure 2. X-ray diffraction analysis of (a) BCP powder and (b) Gel-Pec-BCP scaffold. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Furthermore, the inserted EDS data [Figure 1(c)], taken from the point marked in Figure 1(b), show that the hydrogel scaffold consisted of C, O, Ca, and P from the elements of the gelatin(Gel), pectin, and BCP powders. These data showed that the surface microstructure of the scaffold was composed of BCP powder. In addition, the BCP powder was also dispersed throughout the scaffold surface, which was represented by the white contrast region. The inserted EDS profile [Figure 1(d)] revealed the atomic percentages of Ca and P to be 5.74% and 3.92% (Ca/P ratio = 1.46), respectively.

The crystallographic structure of the minerals was examined by XRD. Figure 2 shows the XRD profiles of the three types of hydrogel scaffolds, including the Gel-Pec-BCP, Gel-Pec-BCP-BMP-2, and Gel-Pec-BCP-VEGF scaffolds, as well as BCP powder. The synthesized powder showed mixed phases of α-TCP, β-TCP, and Hap.[42] Hydrogel scaffolds with broad diffraction peaks, which are the typical XRD pattern characteristic of gelatin at $2\theta = 21.3°$, are shown in Figure 2.[44,45]

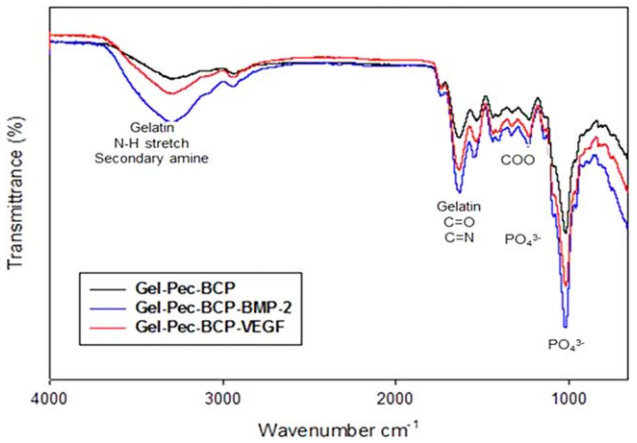

Figure 3. Fourier transform infrared analysis of Gel-Pec-BCP, Gel-Pec-BCP loaded with BMP-2, and Gel Pec-BCP loaded with VEGF scaffolds. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FTIR analysis was carried out to further illustrate the chemical interaction between HA and the Gel-Pec network matrix. Figure 3 shows the FTIR spectra of the three types of samples. The FTIR spectrum of the Gel-Pec-BCP hydrogel showed the presence of a PO_4^{3-} band. The strongest bands were for phosphates, which appeared at 1050 cm^{-1}.[40] The FTIR spectrum of the gelatin(Gel) showed bands at 3443 cm^{-1} due to N—H stretching of an amide bond, and C═O stretching at 1640 cm^{-1}.[44] The pectin spectrum indicated a peak at 3400 cm^{-1} because of the stretching of —OH groups, while the peak at 2913 cm^{-1} was indicative of C—H stretching vibrations. The peak at 1742 cm^{-1} indicated C═O stretching vibrations due to the presence of a $COOCH_3$ group. The peaks at 1441 and 1342 cm^{-1} could be attributed to CH_2 scissoring and —OH bending vibrations, respectively. Finally, the peak at 1150 cm^{-1} suggested the presence of a CH—OH group.[45,46] In comparison with the spectra of Gel-Pec-BCP, these results confirmed the successful reaction among Gel-Pec-BCP, BMP-2, and VEGF.

In Vitro BMP-2 and VEGF Release Test

Figure 4(a,b) shows the BMP-2 and VEGF release profiles from the Gel-Pec-BCP-BMP-2 and Gel-Pec-BCP-VEGF scaffolds, which demonstrated sustained release for up to 28 days. The sustained release may be attributed to electrical interaction between the positively charged amino acid residues in the BMP-2 and VEGF proteins, and the negatively charged sites of the pectin, gelatin hydrogel, and BCP nanopowders.[47] The release profiles of BMP-2 and VEGF from Gel-Pec-BCP also confirmed the strong interaction. In addition, it was confirmed that composite scaffolds had good properties, with improvement of the mechanical strength by polymers, while polymer or ceramic alone are weak. After incorporation of ceramic biomaterials inside the scaffold, they can provide integrity for the framework of the scaffold.[48,49] Moreover, ceramic materials such as HAp[47] and BCP[49] can increase the biocompatibility of the scaffold, because of the similar structure of HAp with bone, also helping to control the release of BMP-2 or VEGF from the scaffolds. The most important factor to consider in the composite system is the achievement of efficient drug delivery for bone regeneration,[49,50] which makes it important to sustain the release of growth factors. In our case, a total of 4.64 ± 0.45% of the BMP-2 was released from the Gel-Pec-BCP-BMP-2 scaffolds on the first day, while a total of 2.07 ± 0.20% of the VEGF was released from the Gel-Pec-BCP-VEGF scaffolds. Steady and slow release was maintained over the next 3 days, with a VEGF release rate of about 2% per day, which was two times less than that of BMP-2. Beginning on day 3, the release rates of BMP-2 were significantly reduced to 1%, which was maintained until day 28. About 17% had been released by the end of the experiment. In contrast, the amount of VEGF released doubled from 5.63 to 10.07%, reaching a total of 67.40% by the end of the experiment. Scaffold preparation should translate to lower costs, and the cost can be decreased by controlled release from the scaffold. This makes the preparation of a scaffold with suitable release characteristics highly important.[48] Depending on the materials chosen for drug or growth factor release, different release profiles can be obtained. For example, in the case of

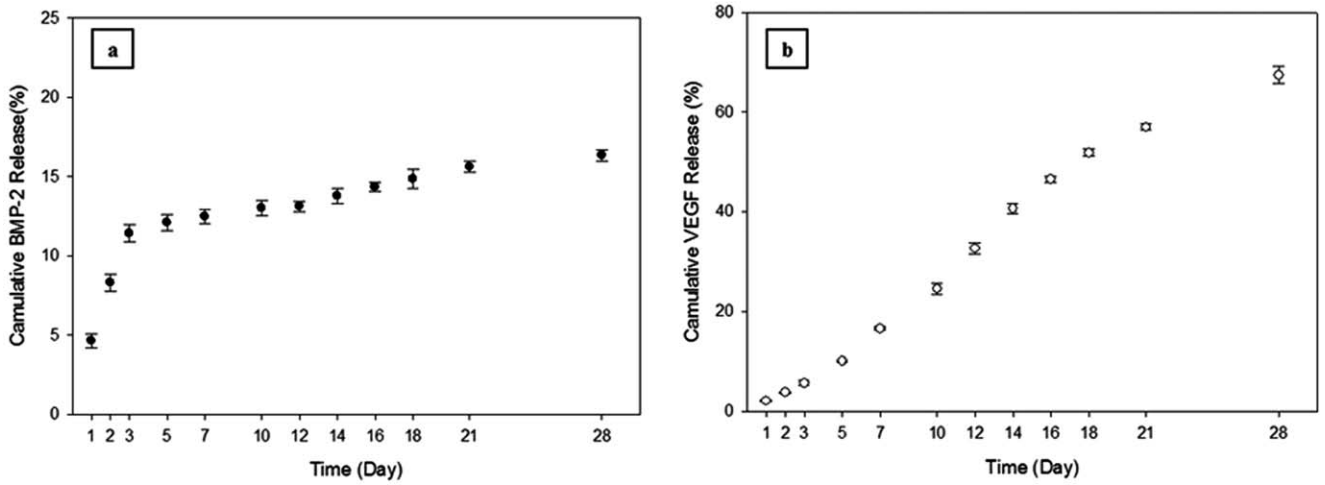

Figure 4. Cumulative release profiles of BMP-2 and VEGF from (a) Gel-Pec-BCP-BMP-2 and (b) Gel-Pec-BCP-VEGF scaffolds. Error bars represent mean ± standard deviation.

BMP-2 loaded into collagen,[51] a burst of release can be observed during 2 days, resulting in release of 70% of the total BMP-2 loaded. In another study done by Brown et al. they report that 92% of the total BMP-2 was released on the first day, while 100% had been released by the end of the 2nd day.[52] This can be related to the fast degradation of collagen during *in vitro* and *in vivo* experiments, which resulted in fast release of the growth factors, like BMP-2, from the matrix. However, other studies reported that a strong interaction between VEGF and collagen was expected, allowing for a very low release of VEGF at the boundary of the matrix, totally about 16% in 10 days.[53] In our study, similar behavior was observed, but at a slightly higher rate of about 24.59%.

In our system, Gel-Pec-BCP acted as a good carrier for growth factors, such as BMP-2 and VEGF, because it was made of a combination of three materials, each with a good effect on the sustained release of growth factors during *in vitro* experiments.

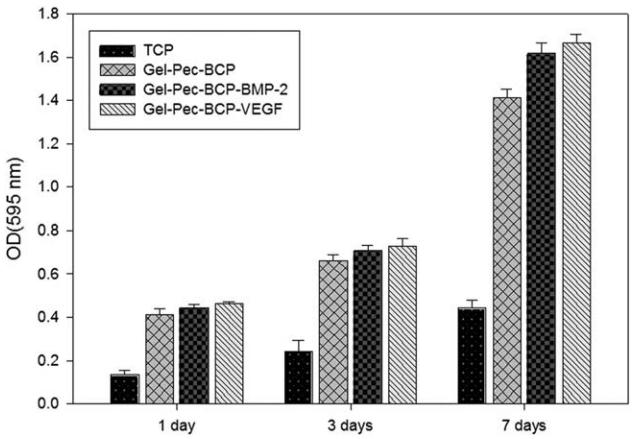

Figure 5. Cell viability on Gel-Pec-BCP, Gel-Pec-BCP-BMP-2, and Gel-Pec-BCP-VEGF crosslinked scaffolds after 1, 3, and 7 days, as observed by the MTT assay.

This was derived from the special advantages of the composite system of gelatin, pectin, and BCP for carrying drugs. In addition, the degree of crosslinking and crosslinking time are also very important for drug release. As mentioned in the experimental methods, the same crosslinking procedure was performed about 2 times for 24 h for all of the types of samples, with and without BMP-2 and VEGF. In previous studies using different types of crosslinkers, the dose (concentration) and time of crosslinking degradation were examined, along with the drug release. In a study done by Vandelli et al., it was shown that by applying the same type of crosslinker and different types of crosslinking, the amount of drug release could change.[54] The use 2% D,L-glyceraldehyde for clonidine hydrochloride gelatin microspheres at a concentration of 2% as a function of time for 1 and 24 h was examined. They showed a decrease of the free amino groups of gelatin after treatment with glyceraldehyde.[54] With increasing the time of crosslinking from 1 to 24 h, the gelatin microsphere crosslinked with 2% (w/v) of glyceraldehyde showed a loss of 30–60% of the amino acids, and a subsequent decrease in the drug release from 40% to 29%.[54] The independent condition of gelatin crosslinking, which demonstrated rapid drug release in a short time, will occur, and after the second crosslinking reaction, drug release will become more prolonged, accompanied with a slower release profile.[54] Therefore, optimization of the crosslinking of scaffold is also an important factor, and affects the sustained, slower release of growth factors. In fact, suitably crosslinked gelatin can act as good candidate for the release of VEGF.

Cell Viability

Viability of MC3T3-E1 cells on the Gel-Pec-BCP, Gel-Pec-BCP-BMP-2, and Gel-Pec-BCP-VEGF scaffolds was measured by the MTT assay at 1, 3, and 7 days (Figure 5). A higher percentage of viable cells was found in the scaffolds loaded with VEGF and BMP-2 than in the scaffolds without growth factors and the TCP (tissue culture plate) group. The quantitative proliferation rate was examined at 1, 3, and 7 days, and gradual increases in

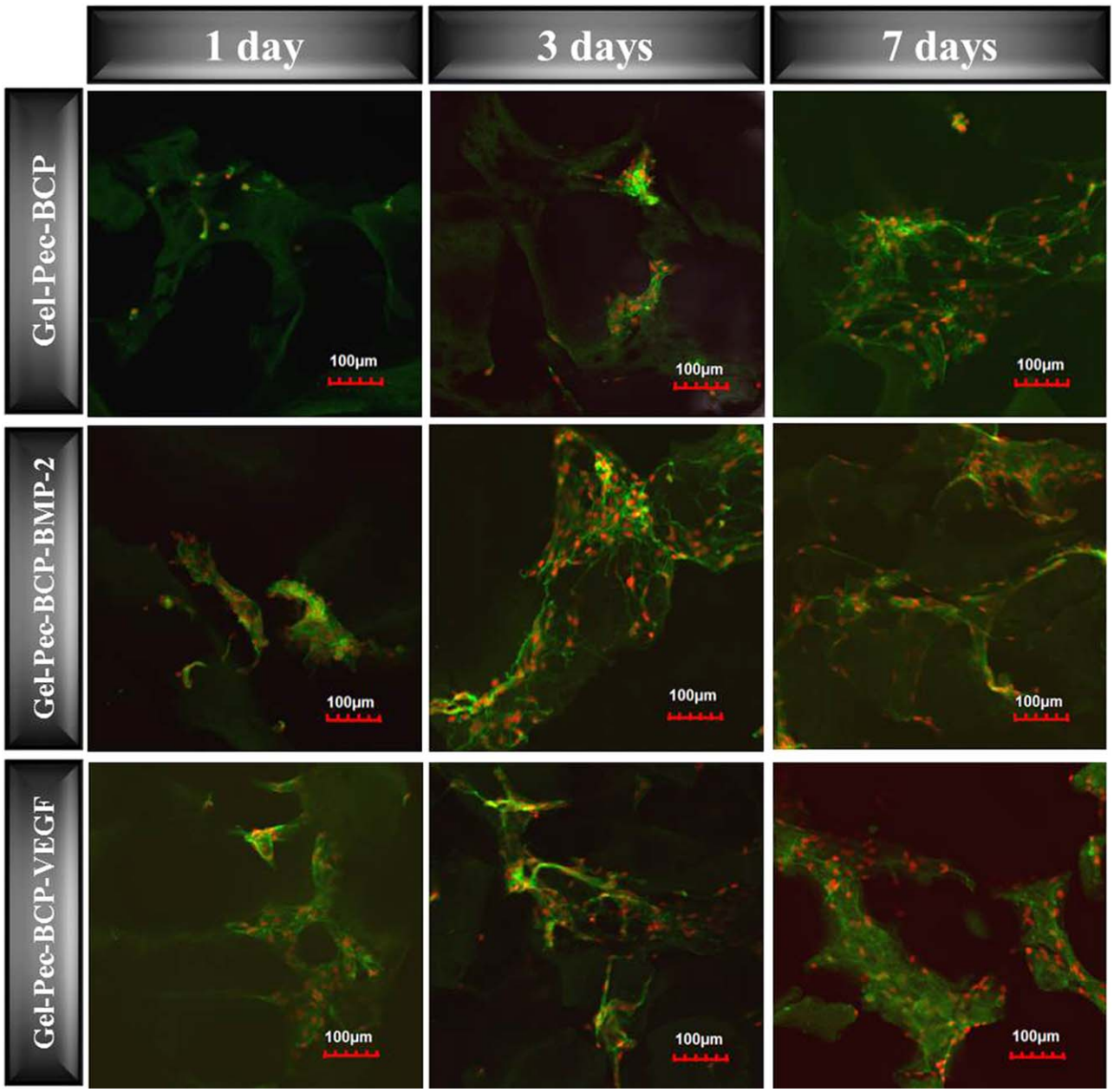

Figure 6. Cell spreading on the Gel-Pec-BCP, Gel-Pec-BCP-BMP-2, and Gel-Pec-BCP-VEGF crosslinked scaffolds after 1, 3, and 7 days, as observed by confocal microscopy. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the cell proliferation rates were observed for all the samples over time, as shown in Figure 5. Cell proliferation differed on day 1 between the Gel-Pec-BCP containing BMP-2 and VEGF scaffolds, and the Gel-Pec-BCP scaffolds. No difference in cell proliferation was observed between the BMP-2 loaded Gel-Pec-BCP scaffolds and the nonloaded Gel-Pec-BCP scaffolds after 3 days, but differences in cell proliferation were detected between the Gel-Pec-BCP loaded with VEGF and the nonloaded Gel-Pec-BCP scaffolds.

Acceptable cell viability was observed for all the composite scaffolds, including Gel-Pec-BCP and Gel-Pec-BCP loaded with BMP-2 and VEGF. These results confirmed the biocompatibility of Gel-Pec-BCP and Gel-Pec-BCP with BMP-2 and VEGF hydrogel scaffolds, and no cytotoxicity was found under any of the conditions. These results suggest that biocompatible and bioactive Gel-Pec-BCP may be applicable for a variety of bone applications and drug delivery.

Cell Proliferation

Figure 6 shows the morphology of the MC3T3-E1 preosteoblasts (1×10^5 cell/well) that had adhered and spread on the hydrogels. Qualitative proliferation and cell morphology on each type of hydrogel scaffold at different time intervals was visualized by staining and fixing the cells with FITC and Hoechst 33342, demonstrating a good match with quantitative data that was

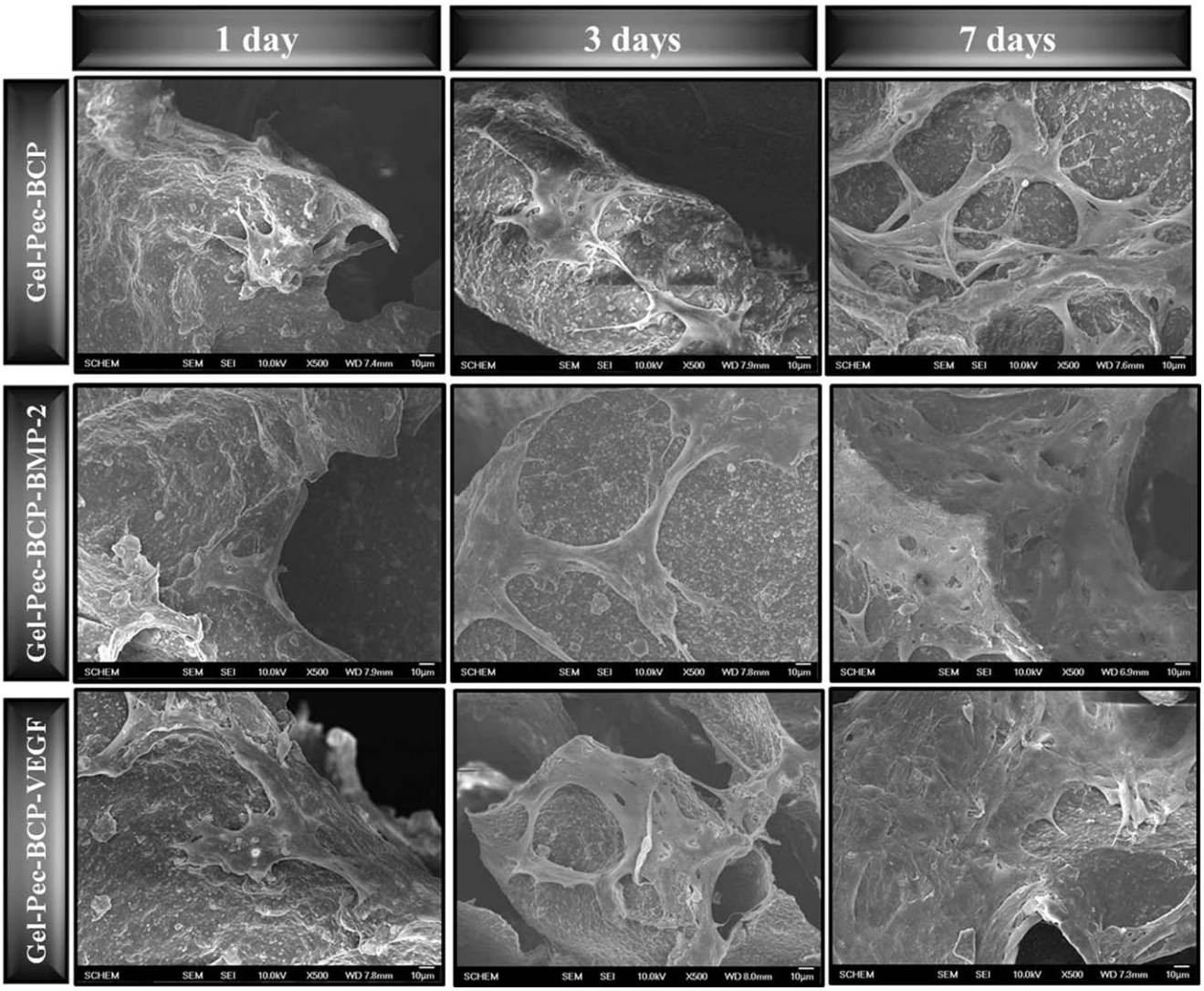

Figure 7. Cell spreading on the Gel-Pec-BCP, Gel-Pec-BCP-BMP-2, and Gel-Pec-BCP-VEGF crosslinked scaffolds after 1, 3, and 7 days, as observed by SEM images.

shown in Figure 5. The staining results showed various cell responses to the hydrogel and hydrogels loaded with BMP-2 and VEGF scaffolds. Both the Gel-Pec-BCP-BMP-2 and Gel-Pec-BCP-VEGF scaffolds showed more rapid cell spreading after 1, 3, and 7 days than that on the Gel-Pec-BCP scaffolds. The higher amount of cells adhered to spread on the Gel-Pec-BCP-BMP-2 and Gel-Pec-BCP-VEGF scaffolds were observed by confocal microscope (Figure 6) and SEM (Figure 7). Therefore, the hydrogels containing BMP-2 and VEGF more effectively promoted cell adhesion, while all the hydrogels were biocompatible. VEGF is the main angiogenic factor that contributes to postnatal neovascularization.[12,17–19,55–57]

One group in Rice University examined the effectiveness of the combination of Gelattin and calcium phosphate, which is also an injectable composite.[48] Furthermore, it exhibited bone regeneration in rabbits. This system shows a beneficial drug-release profile. VEGF interacts synergistically with osteogenic proteins to promote bone formation by prolonging cell survival, inducing osteoblast proliferation and differentiation, and promoting the migration of osteoblasts.[20] In addition, BMPs play key roles inducing bone and cartilage formation.[10] Both growth factors showed acceptable bioactivity after 1 and 3 days, according to their release from the scaffolds. We conclude that the amount of BMP-2 and VEGF released had direct effects on the viability and proliferation of the cells on these scaffolds.

CONCLUSION

Gel-Pec-BCP, BMP-2 loaded Gel-Pec-BCP and VEGF loaded Gel-Pec-BCP scaffolds were successfully fabricated using a freeze-drying technique. The SEM micrographs showed highly interconnected pores of 100–300 μm in the scaffolds. The scaffolds were demonstrated controlled release of BMP-2 and VEGF, and were confirmed to have good sustained release properties. The *in vitro* experiments with the scaffolds containing growth factors showed higher cell proliferation compared to those without growth factors. These data demonstrated that Gel-Pec-BCP,

BMP-2 loaded Gel-Pec-BCP and VEGF loaded Gel-Pec-BCP scaffolds were nontoxic, and provided good preosteoblast spreading behavior, making them promising candidates as bone scaffolds.

ACKNOWLEDGMENTS

This work was supported by- Mid-carrer Researcher program through NRF grant funded by MEST, and partially supported by Soonchunhyang university research fund. The authors are grateful to Mr Hyeong Seok Kim for providing us with nano BCP powder and important guidelines and suggestion.

REFERENCES

1. Oryan, A. A.; Alidadi, S.; Moshiri, A.; Maffuli, N. *J. Orthop. Surg. Res.* **2014**, *9*, 1.
2. Son, J. S.; Appleford, M.; Ong, J. L.; Wenke, J. C.; Kim, J. M.; Chio, S. H.; Oh, D. S. *J. Controlled Release.* **2011**, *153*, 133.
3. Shu, C.; Wenjuan, Zh.; Xu, G.; Wei, Zh.; Wei, J. Dongmei, W. *Mater. Sci. Eng. C.* **2010**, *30*, 105.
4. Karp, J. M.; Dalton, P. D.; Shoichet, M. S. *Scaffolds Tissue Eng. MRS Bull.* **2003**, *28*, 301.
5. Langer, R. *Acc. Chem. Res.* **1999**, *33*, 94.
6. Tessmar, J. K.; Göpferich, A. M. *Adv. Drug Deliv. Rev.* **2007**, *59*, 274.
7. Censi, R.; Di Martino, P.; Vermonden, T.; Hennink, W. E. *J. Controlled Release* **2012**, *161*, 680.
8. Malafaya, P. B.; Silva, G. A.; Baran, E. T.; Reis, R. L. *Curr. Opin. Solid State Mater. Sci.* **2002**, *6*, 283.
9. Torres, A. L.; Gaspar, V. M.; Serra, L. R.; Diogo, G. S.; Fradique, R.; Silva, A. P.; Correia, I. J. *Mater. Sci. Eng. C.* **2013**, *33*, 4460.
10. Adachi, T.; Osako, Y.; Tanaka, M.; Hojo, M.; Hollister, S. L. *Biomaterials* **2006**, *27*, 3964.
11. Chen, Q.-Z.; Thouas, G. A. *Acta Biomater.* **2011**, *7*, 3616.
12. Kempen, D. H. R.; Lu, L.; Heijink, A.; Hefferan, T. E.; Creemers, L. B.; Maran, A.; Yaszermski, M. J.; Dhert, W. J. A. *Biomaterials* **2009**, *30*, 2816.
13. Zhang, W.; Wang, X.; Wang, S.; Zhao, J.; Xu, L.; Zeng, D.; Chen, J.; Zhang, Z. H.; Kaplan, D. L.; Jiang, X. *Biomaterials* **2011**, *32*, 9415.
14. Priddy, L. B.; Chaudhuri, O.; Stevens, H. Y.; Krishnan, L.; Uhrig, B. A.; Willett, N. J.; Guldberg, R. E. *Acta Biomater.*, in press.
15. Kirby, G. T. S.; White, L. J.; Rahman, C. V.; Cox, H. C.; Qutachi, O.; Rose, F. R. A. J.; Hurtmacher, D. W.; Shakesheff, K. M.; Woodruff, M. A. *Polymer.* **2011**, *3*, 571.
16. Hsieh, C.-Y.; Hsieh, H.-J.; Liu, H.-M.; Wang, D.-M.; Hou, L.-T. *Dental Mater.* **2006**, *22*, 622.
17. Luo, T.; Zhang, W.; Shi, B.; Cheng, X.; Zhang, Y. *Clin. Oral Implants Res.* **2012**, *23*, 467.
18. Kim, J.-E.; Kang, S.-S.; Chio, K.-H.; Shim, J.-S.; Jeong, C.-M.; Shin, S.-W.; Huh, J.-B. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol.* **2013**, *115*, e24.
19. Wang, L.; Fan, H.; Zhang, Z.-Y.; Lou, A.-J.; Pei, G.-X.; Jiang, S.; Mu, T.-W.; Qin, J.-J.; Chen, S.-Y.; Jin, D. *Biomaterials* **2010**, *31*, 9452.
20. Lacroix, J.; Jallot, E.; Lao, J. *Chem. Eng. J.*, **2014**, *256*, 9.
21. Linh, N. T. B.; Lee, B.-T. *J. Biomater. Appl.* **2012**, *27*, 255.
22. Bhakta, G.; Rai, B.; Lim, Z. H. H.; Hui, J. H.; Stein, G. S.; Van Wijnen, A. J.; Prestwich, G. D.; Cool, S. M. *Biomaterials* **2012**, *33*, 6113.
23. Smistad, G.; Bøyyum, S.; Alund, S. J.; Samuelsen, A. B. C.; Hiorth, M. *Carbohydr. Polym.* **2012**, *90*, 1337.
24. Kumar, P. T. S.; Ramya, C.; Jayakumar, R.; Nair, S. K. V.; Lakshamanan, V.-K. *Colloids Surf. B: Biointerfaces.* **2013**, *106*, 109.
25. Liu, L.; Fishman, M. L.; Kost, J.; Hicks, K. B. *Biomaterials* **2003**, *24*, 3333.
26. Itoh, K.; Yahaba, M.; Takahashi, A.; Tsuruya, R.; Miyazaki, S.; Dairaku, M.; Togashi, M.; Mikami, R.; Attwood, D. *Int. J. Pharma.* **2008**, *356*, 95.
27. Ahrabi, S. F.; Madsen, G.; Dyrstad, S. A.; Sande, S. A.; Graffner, C. *Eur. J. Pharma. Sci.* **2000**, *10*, 43.
28. Zhao, L.; Gwon, H.-J.; Lim, Y.-M.; Nho, Y.-C.; Kim, S. Y. *Carbohydr. Polym.* **2014**, *102*, 598.
29. Tao, Y.; Tong, X.; Zhang, Y.; Lai, J.; Huang, Y.-R.; Guo, B.-H. *Acta Biomater.* **2013**, *9*, 5022.
30. Rose, J.; Pacelli, S.; Haj, H.; Hopkinspon, A.; White, L.; Rose, F. *Materials* **2014**, *7*, 3106.
31. Gorgieva, S.; Kokol, V. In Hand book of Biomaterials Applications for Nanomedicine; Pignatello, R., Ed.; InTech: Croatia, **2011**, Chapter 2, pp 17–53.
32. Linh, N. T. B.; Min, Y.; Lee, B.-T. *J. Mater. Sci.* **2013**, *48*, 4233.
33. Lynn, A. K.; Yannas, I. V.; Bonfield, W. *J. Biomed. Mater. Res. Part B: Appl. Biomater.* **2004**, *71B*, 343.
34. Moreira, R. B.; Teixeira, J. A.; Furuyama-Lima, A. M.; Souza, N. C. D.; Siqueira, A. B. *Thermochim. Acta.* **2014**, *590*, 100.
35. Mishra, R. K.; Banthia, A. K.; Majeed, A. B. A. *Asian J. Pharma. Clin. Res.* **2012**, *5*, 1.
36. Munarin, F.; Tanzi, M. C.; Petrini, P. *Int. J. Biol. Macromol.* **2012**, *51*, 681.
37. Kim, M.; Franco, R. A.;Lee, B.-T. *J. Eur. Ceram. Soc.* **2011**, *31*, 1541.
38. Bao, T.-Q.; Franco, R. A.; Lee, B.-T. *Biochem. Eng. J.* **2012**, *64*, 76.
39. Bakhtiari, L.; Rezaie, H. R.; Hosseinalipour, S. M.; Shokrgozar, M. A. *Ceram. Int.* **2010**, *36*, 2421.
40. Yang, C. R.; Wang, Y. J. Chen, X. F.; Zhao, N. R. *Mater. Lett.* **2005**, *59*, 3635.
41. Linh, N. T. B., Min, Y.; Lee, B.-T. *Tissue Eng. Part A*, Under review.
42. Lee, B.-T.; Youn, M.-H.; Paul, R. K.; Lee, K.-H.; Song, H.-Y. *Mater. Chem. Phys.* **2007**, *104*, 249.
43. Jones, A. C.; Milthorpe, B.; Averdunk, H.; Limaye, A.; Senden, T. J.; Sakellariou, A.; Sheppard, A. P.; Sok, R. M.;

Knackstedt, M. A.; Brandwood, A.; Rohner, D.; Hutmacher, D. W. *Biomaterials* **2004**, *25*, 4947.

44. Gautam, S.; Dinda, A. K.; Mishra, N. C. *Mater. Sci. Eng. C.* **2013**, *33*, 1228.

45. Dong, Z.; Wang, Q.; Du, Y. *J. Membr. Sci.* **2006**, *280*, 37.

46. Kalapathy, U.; Proctor, A. *Food Chem.* **2001**, *73*, 393.

47. Crouzier, T.; Saihan, F.; Becquart, P.; Logeart-Avramoglou, D.; Picart, C. *Biomaterials* **2011**, *32*, 7543.

48. Peters, J.; Nano, J. D. University of Pittsburgh Swanson School of Engineering. **2013**.

49. Geuze, R. E.; Theyse, L. F. H.; Kempen, D. H. R.; Hazewinkel, H. A. W.; Kraak, H. Y. A.; Oner, F. C.; Dhert, W. J. A.; Alblas, J. *Tissue Eng. Part A* **2012**, *18*, 10.

50. Wernike, E.; Montijovent, M.-A.; Liu, Y.; Wismeijer, D.; Hunzikar, E. B.; Siebenrock, K.-A.; Hofstetter, W.; Klenke, F. M. *Eur. Cells Mater.* **2010**, *19*, 30.

51. Jung, S.-Y.; Ko, Y.-J.; Jang, H.-S.; Kang, S.-W.; Park, J.-H. *Tissue Eng. Regener. Med.* **2013**, *10*, 341.

52. Brown, K. V.; Li, B.; Guda, T.; Perrien, D. S.; Guelcher, S. A.; Wenke, J. C. *Tissue Eng. Part A.* **2011**, *17*, 1735.

53. Angelo, I. D.; Ungaro, F.; Oliviero, O.; Quaglia, F. Netti, P. A. *Biomater. Drug Deliv. Tissue Eng.* **2012**.

54. Vandelli, M. A.; Rivasi, F.; Guerra, P.; Forni, F.; Arletti, R. *Int. J. Pharma.* **2001**, *215*, 175.

55. Roskoski R. Jr. *Crit. Rev. Oncol. Hematol.* **2007**, *62*, 179.

56. Mostefai, H. A.; Andriantsitohaina,V.; Martinez, M. C. *Physiol. Res.* **2008**, *57*, 56.

57. Masuda, H.; Asahara, T. *Cardiovasc. Res.* **2003**, *58*, 390.