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# Heparin-functionalized chitosan–alginate scaffolds for controlled release of growth factor

Yi-Cheng Ho[a], Fwu-Long Mi[a,*], Hsing-Wen Sung[b], Pi-Li Kuo[a]

[a] Department of Biotechnology, Vanung University, Chung-Li 320, Taiwan, ROC
[b] Department of Chemical Engineering/Bioengineering Program, National Tsing Hua University, Hsinchu, Taiwan, ROC

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ABSTRACT

Controlled long-term release of basic fibroblast growth factor (bFGF) has shown a combined effect on the stimulation of regenerating a number of tissues including cartilage, nerve, skin and liver. In this study, three-dimensional scaffolds prepared from the polyelectrolyte complexes (PEC) of chitosan and alginate were developed for the delivery of bFGF. The bFGF-binding efficiency of the chitosan–alginate PEC scaffold, after being conjugated with high concentration of heparin (83.6 μg/mg scaffold), was increased up to 15 times higher than that of original scaffold (65.6 ng bFGF/mg scaffold vs. 4.5 ng bFGF/mg scaffold). The release of bFGF from the original scaffold was quick and the initial burst release was obvious. By functionalizing the scaffold with various concentrations of heparin (17.6 μg, 50.3 μg and 83.6 μg heparin/mg scaffold), the rate of bFGF release from the scaffold decreased in a controlled manner with reduced burst effect. The released bFGF retained its biological activity as assessed by the *in vitro* proliferation of human foreskin fibroblast (HFF). This study shows that a novel bFGF delivery system using the heparin-functionalized chitosan–alginate PEC scaffold exhibits controllable, long-term release of bFGF and could prevent the growth factor from inactivation.

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## 1. Introduction

In recent years there has been increasing interest in optimizing engineered tissue regeneration using biocompatible and biodegradable materials, with the capability to release growth factors in a controlled manner (Langer and Vacanti, 1993; Langer and Tirrell, 2004; Beng et al., 2007). Tissue regeneration is a complex biological process that involves chemotaxis and division of cells, neovascularization and synthesis of extracellular matrix (ECM) proteins. Polypeptide growth factors such as basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β) appear to play important roles in initiating and sustaining the phases of tissue repair (Giannoni and Hunziker, 2003). The growth factors initiate their effects by binding to an activating specific, high-affinity receptor proteins located in the plasma membrane of target cells (Chua et al., 2004). Activation of the receptors eventually results in stimulating a number of processes, including those involved in wound healing (Martin et al., 1998).

As is widely recognized, bFGF is a heparin-binding growth factor that promotes proliferation of fibroblasts, neovascularization, osteogenesis, and nerve regeneration (DiGabriele et al., 1998). Such a strong affinity to heparin is helpful to protect bFGF from denaturation and enzymatic degradation (Vemuri et al., 1994). Therefore, heparin in the ECM plays a major role in storing growth factors, slowing their release while retaining their bioactivity (Baskin et al., 1989). Several approaches has been used to combine heparinized biomaterials with the growth factors and to accomplish sustained release of heparin-binding growth factor with biological activity. bFGF was incorporated into heparined poly(lactic-co-glycolic acid) (PLGA) microspheres, fibril gels and collagen matrices to be administered locally or directly at the wound or tissue defect site (Jeon et al., 2005; Pike et al., 2006; Lee et al., 2008).

Chitosan and alginate are respectively a cationic and anionic polysaccharide in nature. Microcapsules or beads of chitosan–alginate polyelectrolyte complexes (PEC) have been prepared for controlled drug release and tissue engineering (Xu et al., 2007; Babister et al., 2008). Therefore, chitosan and alginate were selected to design a novel, three-dimensional (3-D), heparin-functionalized scaffold to release bFGF with biological activity. This heparinized scaffold in contrast to traditionally used hydrogels might provide macropores for cell adhesion and cell growth and might be beneficial to tissue regeneration. The aim of present study was to examine the effect of immobilized heparin on the stability of scaffolds and the bFGF-binding and release properties. Biological activities of the bFGF released from the heparin-functionalized scaffold were also investigated.

* Corresponding author. Tel.: +886 3 4515811x56666; fax: +886 3 4333063.
*E-mail address:* flmi530326@vnu.edu.tw (F.-L. Mi).

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## 2. Experimental

### 2.1. Materials

Chitosan (MW ~$3.0 \times 10^5$) with a degree of deacetylation of approximately 85% were acquired from Fluka (Switzerland) and alginate sodium salt of low viscosity (250 cps for a 2% solution at 25 °C) was purchased from Lancaster (England), respectively. Heparin sodium salt (165 units $mg^{-1}$) was obtained from Sigma Chemical Company (USA). Human recombinant bFGF (isoelectric point is 9.6) and ELISA kit were purchased from R&D Ltd. (USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) and all other reagents and solvents used were of reagent grade.

### 2.2. Preparation of the chitosan–alginate PEC gel

The chitosan–alginate PEC gel was prepared using a homogenizing interpolyelectrolyte complex method. Chitosan solution (1.0%, w/v) was prepared by dissolving chitosan powder (2 g) in 200 ml of deionized water containing acetic acid (1.0%, w/v) at room temperature. Alginate solution (1.0%, w/v) was prepared by dissolving sodium alginate powder (2 g) in 200 ml of deionized water at room temperature. The dissolved chitosan solution then was homogenized with alginate solution (blend ratio is 1:1) using a homogenizer (IKA, T25) until a viscose chitosan–alginate PEC gel was derived. The prepared chitosan–alginate PEC gel was cured in 2% (w/v) of $CaCl_2$ aqueous solution for 60 min. The $CaCl_2$-cured gel was washed with deionized water for several times to remove the residual ions.

### 2.3. Preparation of heparin-functionialized chitosan–alginate PEC scaffold

The previously prepared chitosan–alginate PEC gel was functionalized with heparin and was reshaped into scaffolds. Conjugation of heparin with the chitosan–alginate PEC gel was performed with a procedure that was adopted from the literatures (Wissink et al., 2001; Yao et al., 2006). Carboxylic acid groups of heparin (Hep-COOH) were activated using a molar ratio of EDC:Hep-COOH of 0–10.0. To a 2% (w/v) solution of heparin in 0.05 M MES buffer (pH 5.0), EDC and NHS in a molar ratio of 0.6 were added. After 10 min, 0.2 g of chitosan–alginate PEC gel were immersed into this solution. The reaction was allowed to proceed for 4 h at 37 °C and the amount of conjugated heparin was determined using toluidine blue as reported by Hinrichs et al. (1997). Afterward, the heparinized chitosan–alginate PEC gels were extensively washed with deionized water. The gels then were frozen at −80 °C and lyophilized by Eyela, FD-5N (Japan) freeze-drier for the preparation of scaffolds. Chemical structures of the heparinized chitosan–alginate PEC scaffolds were investigated by Fourier transform infrared (FT-IR) spectrometry (PerkinElmer Spectrum RX1 FT-IR System, Buckinghamshire, England).

### 2.4. SEM study

The prepared heparinized chitosan–alginate PEC scaffolds were attached onto a double-sided adhesive tape and fixed to an aluminum stage, respectively. The scaffolds were cut by a razor, and then were sputter-coated with gold in a thickness of $500 \times 10^{-8}$ cm using a Hitachi coating unit (IB-2 coater). Subsequently, the morphologies on surface and cross-section of the scaffolds were examined using a Hitachi S-2300 scanning electron microscopy.

### 2.5. bFGF-binding study

The 10 mg heparinized scaffolds were equilibrated overnight in 5 ml buffered Saline (PBS) at 4 °C. After blotting dry, the scaffolds were incubated with 0.5 ml bFGF solution (5 μg bFGF/ml) in PBS, for 120 min at room temperature. Thereafter, the samples were washed in 10 ml PBS (3 times for 5 min, removing all non-bound bFGF), and the amount of non-bound bFGF in the wash buffer was measured by enzyme-linked immunosorption assay (ELISA) kit (Human bFGF Duoset; R&D Systems, Minneapolis, MN, USA). ELISA plates (Elisa-PS-96A-F-H, Advangene Consumables Inc., USA) were coated with capture monoclonal antibodies, and blocked with 1% BSA (w/v) for 1 h. After adding appropriately the diluted samples to the ELISA plates, bound bFGF was detected using anti-human bFGF polyclonal antibodies. Then, streptavidin-conjugated horseradish peroxidase was added to the plates. The enzyme substrate (tetramethylbenzidine and peroxide) was added and incubated for color development for 20 min. The enzyme reaction was stopped by adding an acidic solution. The absorbance of the samples was read at 450 nm using PowerWave X340 (Bio-TEK Instrument Inc., USA) plate reader. The amount of bFGF was determined from a calibration curve based on known concentrations of bFGF diluted in PBS for 120 min at room temperature. All of the experiments were repeated for five times.

### 2.6. bFGF release experiments

The release profile of bFGF from the scaffolds conjugated with various amounts of heparin was determined to study the effects of heparin on the bFGF release. The samples were incubated at 37 °C under continuous agitation, in 4 ml of PBS (pH 7.4). At various time points, the supernatant was withdrawn and fresh buffer was replenished. The amounts of bFGF in the supernatants were determined with above-mentioned method of ELISA assay. All of the experiments were repeated for five times.

### 2.7. Proliferation assay of human foreskin fibroblasts (HFF)

Before cell testing, all scaffolds were punched into round sections with diameter of 6 mm. The round sections were sterilized using UV lamp for 2 h and placed in the wells of 96-well plates. HFF (Hs68, ATCC) incubated in 1:1 Mixture of Dulbecco's modified Eagle's medium and Nutrient Mixture F-12 (DMEM/F12; Gibco BRL, Grand Island, NY, USA) containing and without containing 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA) was respectively used as a positive control and blank control. The bioactivity of bFGF released from the scaffolds without heparin (original scaffold) or conjugated with high content of heparin (83.6 μg heparin/mg scaffold) were assessed *in vitro* by determining its ability to stimulate the proliferation of HFF cells cultured in a basal medium (2:1 mixture of DMEM/F12 and PBS) without FBS. HFF ($1 \times 10^4$ cells/ml) were seeded in each well containing the scaffold. On days 1–4, viable cell densities in all wells were measured by MTT assay. Cell MTT viability was measured after adding 100 ml MTT solution (5 mg/ml) to every well and culturing for 4 h. To dissolve the formazan pigment 500 ml DMSO was added to the wells. To a 96-well ELISA Plate 200 ml pigment solution was added and the absorbance was measured at 490 nm.

### 2.8. Statistical analysis

Statistical analysis for the determination of differences in the measured properties between groups was accomplished using one-way analysis of variance and determination of confidence intervals, performed with a computer statistical program (Statistical Analysis System, Version 6.08, SAS Institute Inc., Cary, NC). All data are

presented as a mean value with its standard deviation indicated (mean ± S.D.).

## 3. Result and discussion

### 3.1. FT-IR study

Fig. 1 shows the FT-IR spectra of chitosan, alginate and the chitosan–alginate PEC scaffolds. Chitosan displayed the vibrations at 3290 cm$^{-1}$ (O–H and N–H stretch), 2864 cm$^{-1}$ (C–H stretch), 1650 cm$^{-1}$ (amide I), 1597 cm$^{-1}$ (N–H bending of amine II), 1562 cm$^{-1}$ (N–H bending of amine), 1150 cm$^{-1}$ (antisymmetric stretch C–O–C and C–N stretch) and 1026 cm$^{-1}$ (skeletal vibration of C–O stretch) (Fig. 1A). Alginate displayed the vibrations at 3600–3000 cm$^{-1}$ (O–H stretch), 3000–2850 cm$^{-1}$ (C–H stretch), 1621 cm$^{-1}$ (COO$^{-}$ antisymmetric stretch), 1421 cm$^{-1}$ (COO$^{-}$ symmetric stretch) and 1081–1027 cm$^{-1}$ (C–O–C antisymmetric stretch) (Fig. 1B). After mixing alginate with chitosan, superposition of the bands assigned to the carboxylate group on alginate and the amine/amide groups on chitosan resulted in the peak shift (1610 cm$^{-1}$) and the increase in peak intensity (Fig. 1C). This result indicated that the carboxylate groups (–COO$^{-}$) of alginate majorly react with protonated amino (–$NH_3^+$) groups via electrostatic attraction to form interpolyelectrolyte complex. Additionally, the interactions between amide bonds on chitosan and the protonated carboxylate groups on alginate could also form intermolecular hydrogen bonds between the polymers.

The FT-IR spectra of heparinized chitosan–alginate PEC scaffolds is also shown in Fig. 1D. The characteristic absorption bands (S=O asymmetric stretch) in the area of 1160–1260 cm$^{-1}$ for the associated sulfate groups (2-O, 6-O-sulfation and N-sulfation), were attributed to the conjugated heparin. Additionally, the characteristic peak observed at 800 cm$^{-1}$ representing the C–O–S stretch, was also assigned to the 2-O, and 6-O-sulfation on heparin. The bands appeared at 1650 cm$^{-1}$ and 1597 cm$^{-1}$ were attributed to the formation of amide bonds between the amine groups and the carboxyl groups on heparin or alginate. These results suggested that heparin was successfully conjugated to the chitosan–alginate PEC scaffolds.

### 3.2. Characterization of heparinized chitosan–alginate PEC scaffolds

In this study, chitosan–alginate scaffolds were prepared for sustained delivery of bFGF with biological activity. The selection of chitosan and alginate as scaffold components was based on their biocompatible and biodegradable properties and their long history of safe use in biomedical application. bFGF has angiogenic and mitogenic properties, and has demonstrated its stimulative effects in accelerating wound healing processes (Martin et al., 1998). However, it would be necessary to deliver the growth fac-

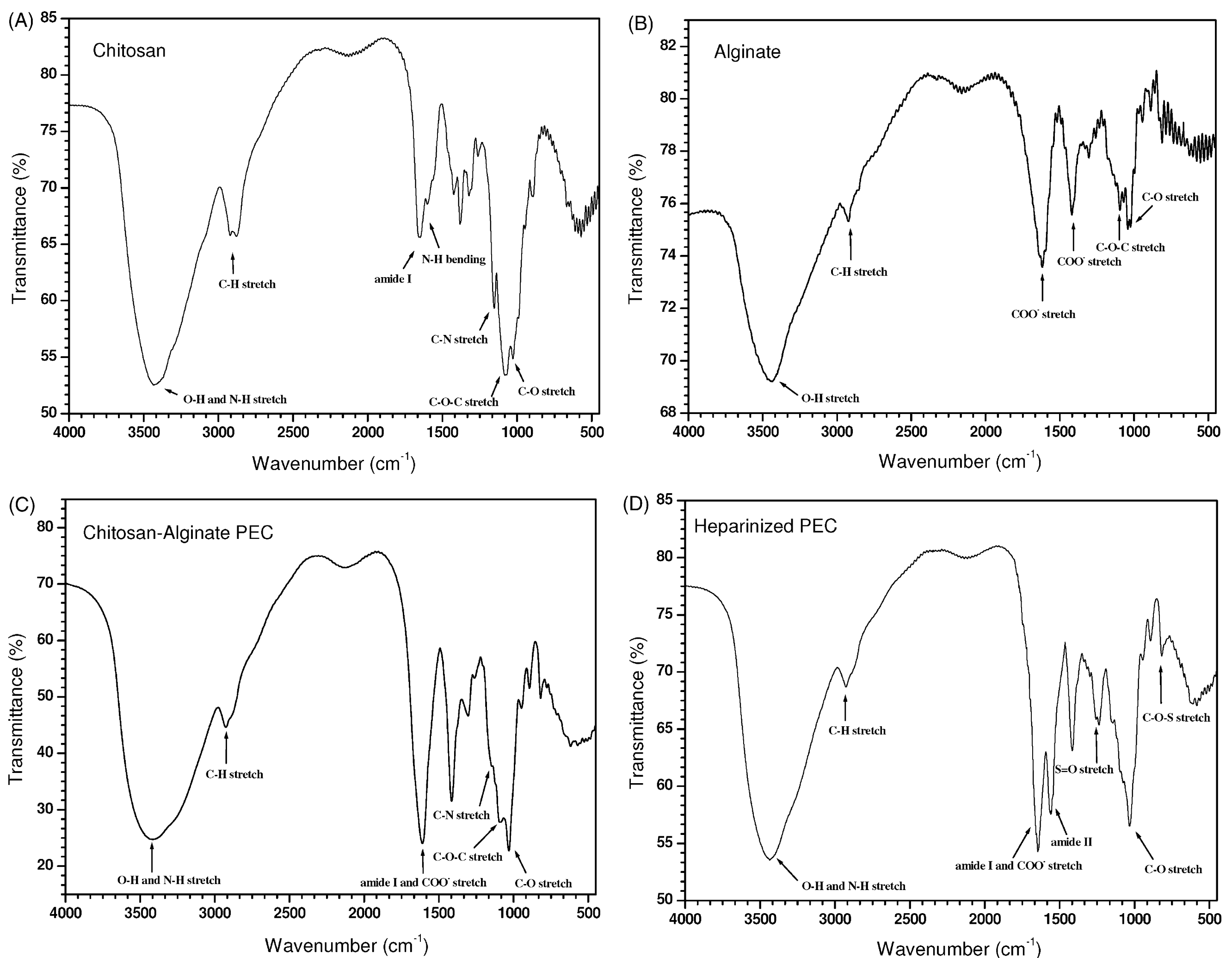


**Fig. 1.** FT-IR spectra of (A) chitosan; (B) alginate; (C) chitosan–alginate PEC; (D) heparinized chitosan–alginate PEC scaffolds.

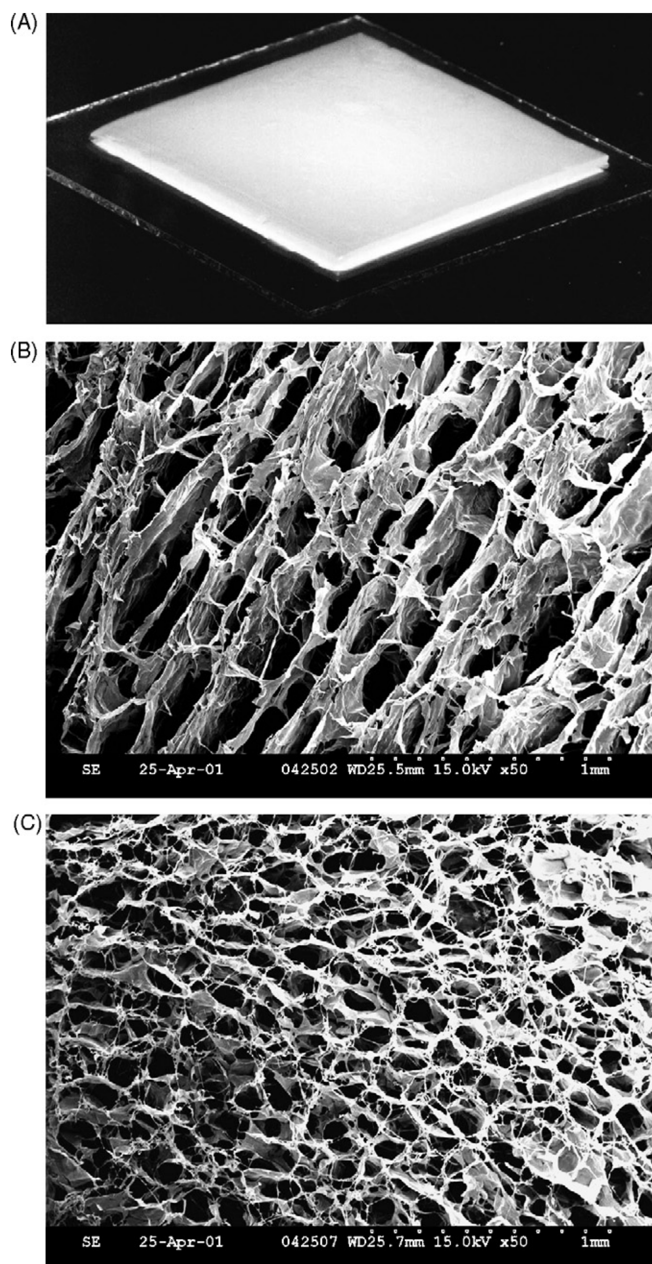


**Fig. 2.** (A) The photograph of heparinized chitosan–alginate PEC hydrogel. SEM micrographs of the heparinized chitosan–alginate PEC scaffolds (B) cross-section; (C) surface.

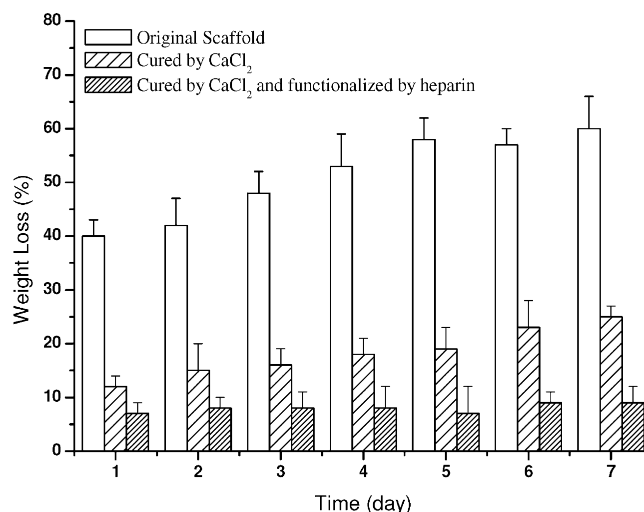


**Fig. 3.** Percentage weight loss of original chitosan–alginate PEC scaffolds, and the scaffolds cured by $CaCl_2$ or functionalized with heparin.

tor in a controlled manner that increases the therapeutic efficacy of bFGF (Cai et al., 2005; Jeon et al., 2005; Mi et al., 2006; Pike et al., 2006; Nillesen et al., 2007). After functionalized with heparin, bFGF might be able to bind with the heparinized chitosan–alginate scaffold and could be released in a sustained way to accelerate the growth of cell into the scaffold, and at the tissue defect site around the scaffold.

Therefore, the properties such as the pore size and the stability of the scaffolds, as well as the amount of conjugated heparin should be characterized. The picture of heparinized chitosan–alginate PEC hydrogel is shown in Fig. 2A. SEM micrographs (surface and cross-section) of the heparinized chitosan–alginate PEC scaffolds are shown in Fig. 2B and C. The lyophilized scaffolds were porous and had pore sizes larger than 100 μm. The 3-D porous chitosan–alginate PEC scaffold was expected to fit the requirement for cell cultures. Fig. 3 showed the stability of the chitosan–alginate PEC scaffolds in PBS buffer, which was investigated by determining the percentage weight loss of the scaffolds. The weight loss of the scaffold after cured with $CaCl_2$ and functionalized with heparin was minimal. This is due to the reason that the carboxylate groups on alginate were ionic-crosslinked with $CaCl_2$, while the carboxylate groups on alginate and heparin could react with the amine groups on chitosan to form amide bonds by using EDC/NHS as a cross-linker (Fig. 4). By means of the curing and cross-linking processes, a scaffold which was stable in PBS could be prepared.

In the conjugation process, carboxylic acid groups of heparin were pre-activated using EDC/NHS, followed by reaction of the activated heparin with the amine groups on chitosan. Using increasing ratios of EDC:Hep-COOH, the covalent bonds introduced between conjugated heparin and chitosan increased. The excess EDC/NHS in the heparine solution could also active the carboxylate groups on alginate to react with chitosan. Therefore, the chain flexibility of chitosan was significantly decreased and further heparin-conjugation reaction would be reduced. As shown in Fig. 5, the amount of conjugated heparin increased with increasing ratios of EDC:Hep-COOH to a maximum of approximately 83.6 μg heparin per mg of the chitosan–alginate PEC scaffold at a molar ratio of EDC:Hep-COOH of 4. The scaffolds conjugated with 83.6 μg, 50.3 μg and 17.6 μg heparin/mg scaffold were selected for the examination of bFGF-binding efficiency.

### 3.3. Binding of bFGF to the scaffold

The growth factor was bound to the heparin-functionalized chitosan–alginate PEC scaffold through the formation of bFGF–heparin complex. The amount of bFGF that could be adsorbed by the heparinized chitosan–alginate scaffolds is shown in Fig. 6. Obviously, the heparinized scaffolds exhibited higher affinity than the chitosan–alginate scaffold alone. The scaffolds distinguished in their ability to bind bFGF depending on the amount of conjugated heparin. The chitosan–alginate scaffold conjugated with 83.6 μg heparin/mg scaffold had a greater ability to bind bFGF (65.6 ng bFGF/mg scaffold), as compared with that conjugated with 50.3 and 17.6 heparin/mg scaffold (42.2 ng bFGF/mg and 23.2 ng bFGF/mg scaffold).

As was reported, depending on molecular weight, a heparin molecule in solution can bind up to 13 molecules of bFGF (Arakawa et al., 1994; Harmer et al., 2004). In this study, 10 mg heparinized scaffolds were incubated with 0.5 ml bFGF solution (5 μg bFGF/ml) in PBS. Thereafter, the samples were washed with

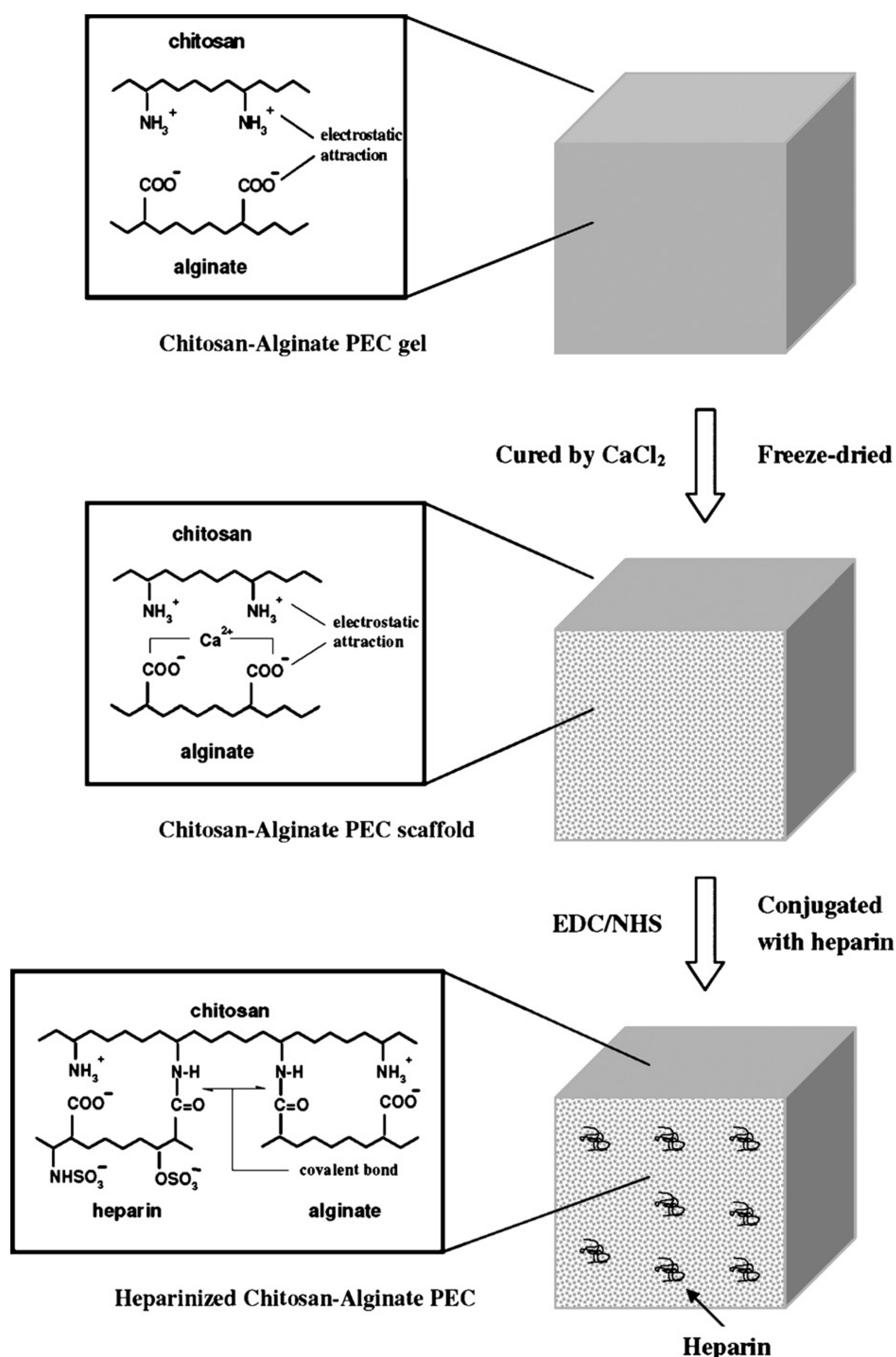


**Fig. 4.** Preparation of the heparinized chitosan–alginate PEC scaffolds by means of the curing and cross-linking processes.

PBS. The bFGF-binding efficiency showed a non-linear correlation with immobilized heparin up to 65.6 ng bFGF/mg scaffold. The result suggested that bFGF was excessive and about 70% of bFGF was not adsorbed by the scaffold. One milligram of immobilized heparin was bound per 0.79–1.32 μg of bFGF, indicating a much lower bFGF-binding efficiency. This was most likely caused by the cross-linking process for the immobilization of heparin to the chitosan–alginate PEC scaffold, which depleted the fractions capable of binding to bFGF (Wissink et al., 2001).

### 3.4. Release of growth factors

The release of growth factor was determined by ELISA assay. Fig. 7 shows the release profile of bFGF from the original and heparinized chitosan–alginate PEC scaffolds. The release of bFGF from the original chitosan–alginate PEC scaffold was more rapid than that from the heparin-functionalized one. Almost all of the bFGF was released from the original chitosan–alginate PEC scaffold within 12 h. To determine whether the release rate can be controlled by functionalizing the chitosan–alginate PEC scaffold with heparin, the rates of bFGF release from the scaffold conjugated with various concentrations of heparin were compared (Fig. 7). The bFGF release rate decreased with the increased amount of heparin. At a low concentration of heparin (17.6 μg/mg scaffold), approximately 82% of the initially loaded bFGF was released within 12 h. In contrast, at a high concentration of heparin (83.6 μg/mg scaffold) in the scaffold, approximately 45% and 84% of the loaded bFGF was released within 12 h and 96 h, respectively.

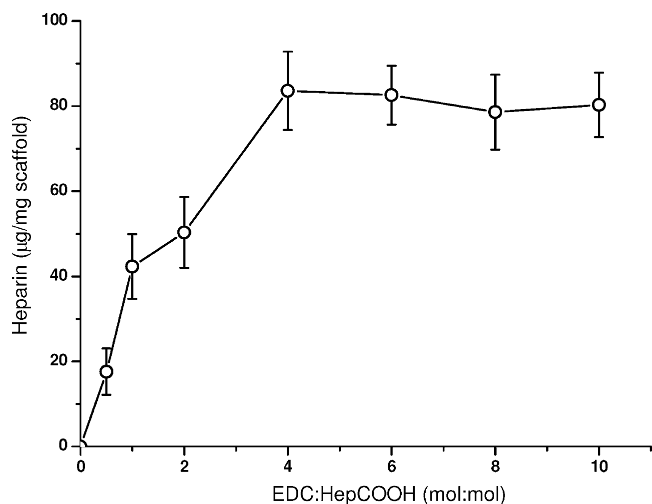


**Fig. 5.** Conjugation of heparin with the heparinized chitosan–alginate PEC scaffolds as a function of the molar ratio of EDC to Hep-COOH used for conjugation. The values represent the mean ± S.D. ($n = 5$).

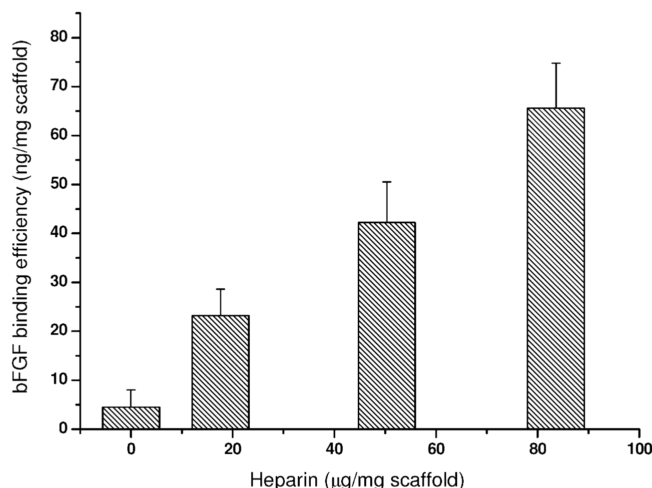


**Fig. 6.** Binding of bFGF to heparinized chitosan–alginate PEC scaffold, as a function of the amount of heparin conjugated. The scaffolds were incubated with 0.5 ml bFGF solution (5 μg bFGF/ml) in PBS, for 120 min. The values represent the mean ± S.D. ($n = 5$).

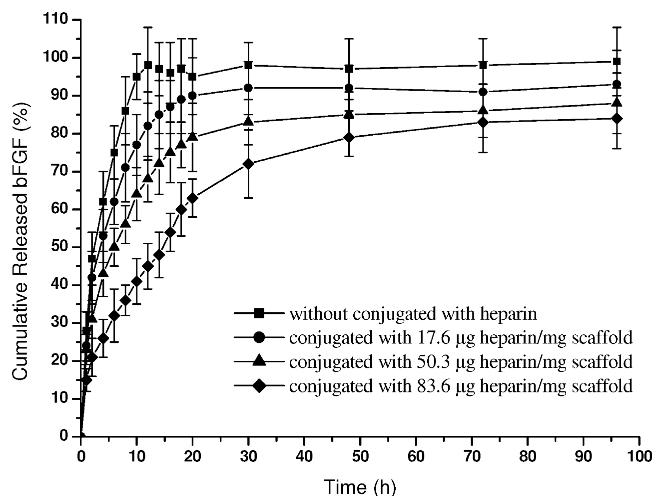


**Fig. 7.** Cumulative release of bFGF from the heparinized chitosan–alginate scaffolds. The values represent the mean ± S.D. ($n = 5$).

It is noted that binding of bFGF and heparin is mediated by ionic interaction between both 2-O-sulfate groups and N-sulfate groups of heparin molecules (Ishihara et al., 1994; Rusnati et al., 1994) and certain lysine ($Lys^+$) and arginine ($Arg^+$) cations in proteins and peptides (Erikson et al., 1991). Multiple clusters of basic amino acids in bFGF that has the proper spacing could result in much stronger binding force with heparin (Fromm et al., 1997). The scaffolds immobilized with more heparin will provide stronger attraction force for closely binding with bFGF. Attributed to the reasons, the release rate of bFGF from the heparinized chitosan–alginate PEC scaffold decreased, and the burst effect could be reduced.

A thermosensitive, injectable tetronic–oligolactide–heparin (TLH) hydrogel has been prepared by coupling heparin to polymerized tetronic–oligolactide for use in improving tissue regeneration (Go et al., 2008). TLH hydrogel released about 40% of the entrapped bFGF over 100 h without a significant initial burst. The growth factor releasing from the heparinized chitosan–alginate PEC scaffold is quicker than its TLH hydrogel counterpart. This is possibly due to the fact that our scaffold contains porous structures differing from the TLH hydrogel scaffold (Fig. 2). Our scaffold might provide macropores for cell adhesion and cell growth and might be beneficial to tissue regeneration. However, the bFGF release rate increased because the porous scaffold lacked the diffusion resistance as compared with the hydrogel systems.

### 3.5. *Biological activity of released bFGF*

Fig. 8 shows the MTT viability of the stimulating effect of bFGF, released from the heparinized chitosan–alginate scaffolds, on the growth of HFF cells. HFF cells cultured in the medium containing and without containing FBS was respectively used as a positive control and blank control. In the absence of FBS, no additional cell growth could be observed. The biological activity of released bFGF was examined by measuring its ability to stimulate the proliferation of HFF cells cultured in medium containing the scaffolds. The culture media was discarded and fresh culture media was fed every 24 h. It was found that the bFGF-containing scaffolds, without functionalized with heparin, could not promote the growth of HFF cells, in comparison to the cells cultured in basal medium (without FBS). In contrast, bFGF released from the chitosan–alginate PEC scaffold functionalized with 83.6 μg heparin/mg scaffold showed the best efficiency for the enhancement of cell-proliferation which is comparable to that of cells cultured in the medium containing FBS (positive control).

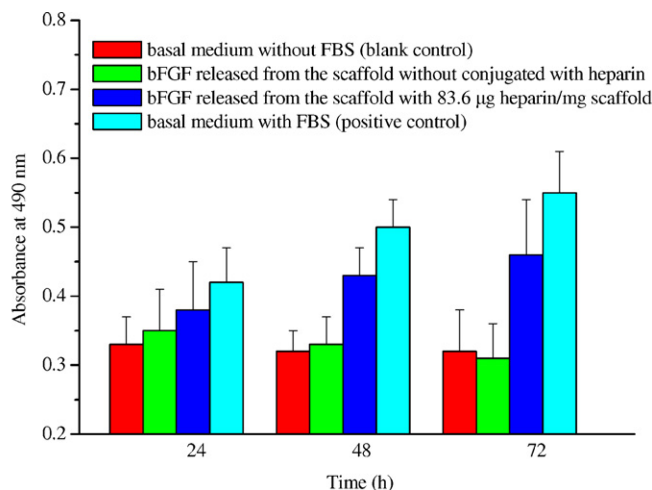


**Fig. 8.** MTT viability of the stimulating effect of bFGF, released from the heparinized chitosan–alginate scaffolds, on the growth of HFF cells. Cell seeding density is $1 \times 10^4$ cells/ml.

bFGF is known as a heparin-binding growth factor, because of its high-affinity for heparin (Yayon et al., 1991). At physiological pH and temperature, the *in vitro* half-life time of bFGF activity is approximately 12 h (Ishihara et al., 1994). Binding of bFGF to heparin induces a conformational change in the bFGF molecule (Prestrelski et al., 1992; Spivak-Kroizman et al., 1994), resulting in an increased resistance against thermal denaturation and enzymatic degradation, and a reduced inactivation at acidic pH (Vemuri et al., 1994; Walker et al., 1994). The result from the cell-proliferation study indicated that bFGF released from the heparinized scaffold retained its biological activity, therefore enhanced the proliferation of HFF cells.

## 4. Conclusion

This study demonstrates the preparation of a three-dimensional (3-D), heparin-functionalized chitosan–alginate PEC scaffold with the ability to control the release of bFGF. The modified method can be used to produce stably immobilized heparin on the 3-D chitosan–alginate PEC scaffold. By binding bFGF with the immobilized heparin, the growth factor was introduced in the scaffold. The release rate can be controlled with the immobilized heparin concentration and the released bFGF from the scaffold retained its biological activity. The released bFGF fraction from the chitosan–alginate PEC scaffold functionalized with 83.6 μg heparin/mg scaffold maintained its activity on the proliferation of HFF cells. The local, controlled long-term delivery system for bFGF developed in this study may be a potential therapeutic method for tissue regeneration.

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