Abstract: Exogenous administration of growth factors has been identified as a potential therapeutic approach for healing wounds. A way to enhance the efficacy of growth factors would be to achieve spatiotemporal control over their delivery to desired sites for an extended period. In this study, we designed and prepared a kind of double-layered collagen membrane, a dense layer and a loose layer, which incorporated basic fibroblast growth factor (bFGF)-loaded chitosan-heparin nanoparticles. The nanoparticles were prepared by polyelectrolyte gelation process and then were sandwiched between the two layers of collagen membrane. The release of model protein human serum albumin (HSA) from the double-layered membrane was tracked by radio-label assay, and the bioactivity of the growth factor on fibroblast cell (L929) was evaluated by MTT assay. The release of protein displayed a spatiotemporal control model and its release in undesired direction was lessened. The bFGF maintained the bioactivities after release from the membranes. Moreover, different release amounts of bFGF from the different layers of the membrane induced significant difference in cell proliferation when the cells were seeded on the different layers of membrane in vitro. This kind of double-layered collagen membrane could have potential applications in the field of tissue repair due to the spatiotemporal control over growth factor delivery, the mild fabrication conditions, and the simple processes.

Key Words: spatiotemporal delivery, growth factor, collagen, double-layered membrane

INTRODUCTION

In general, tissue regeneration displays three overlapping but distinct stages: inflammation, new tissue formation, and remodeling, and is regulated by many growth factors on a temporal and spatial model at the site of injury. Exogenous administration of growth factors has been identified as a potential therapeutic approach for healing acute and chronic wound, but it has been a great challenge to meet the need of temporal and spatial characteristics of those growth factors in tissue repair.

For tissue regeneration, biomaterials and scaffolds have been developed to deliver growth factors. It has been recognized increasingly that these delivery systems should be designed to deliver the growth factors on a spatiotemporal model. "Spatiotemporal delivery" means (i) the released growth factors must target the desired site and cells with spatial gradient, and limit the release to the outside of defects and (ii) their release should be sustained for a relatively long period to obtain the desired effect.

A number of local delivery and spatial gradient systems have been developed for spatial control delivery of growth factors. Previous studies on these systems focused on the local release of growth factors from implanted devices. However, few studies met the challenge to construct scaffolds with spatial concentration gradient. A successful example was reported by Mooney's group. They prepared several scaffolds with layered materials incorporating different amounts of growth factors and found spatially organized tissues appeared due to the spatially localized and temporally controlled properties of the growth factors. It should be noted that the release of growth factors to the outside of defects might induce unpredictable outcomes. Therefore, scaffolds with spatial concentration gradient should further address preventing or reducing delivery into surrounding uninjured areas.

Various methods of delivering growth factors by using materials or scaffolds to achieve sustained release, such as adsorbing growth factors to the materials directly, blending

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microspheres containing growth factor into the matrix, mixing protein powder containing growth factor into the materials or covalently conjugating them to the polymer scaffolds during processing, have been studied. Adsorbing growth factors into the materials has the drawback of low loading efficiency and rapid release. While it was expected to control the release better by incorporating microparticles or nanoparticles with growth factors into biomaterials or scaffolds. The release could be controlled not only by the scaffold itself but also by the degradation and release kinetics of the micro-particles. Hasirci's group developed several 3-D fiber mesh scaffolds using chitosan and chitosan-poly(ethyleneoxide) (PEO), and found the bone morphogenetic proteins (BMPs) retained the bioactivities with different release profiles when BMPs-loaded nanoparticles were incorporated in or on the scaffolds. Their methods achieved prolonged release of BMPs by releasing the growth factor from the scaffold to fiber first, and then to media. Kim et al. constructed novel heparin-conjugated poly(lactide-co-glycolide) (PLGA) nanospheres (HCPNs) for long-term, zero-order delivery of basic Fibroblast Growth Factor (bFGF). The release of bFGF from the HCPNs was sustained for 3 weeks, while the release could be sustained for more than 1 month when the HCPNs were suspended in fibrin gel; and the bFGF release from the fibrin gel was controllable by the fibrinogen concentration. These growth factor delivery systems have proven that the sustained release rate was influenced by the scaffold’s structure when growth factors loaded micro/nano particles were incorporated into the scaffolds. Therefore, it should be feasible to achieve proper release kinetics by adjusting the structure of biomaterials or scaffolds.

Collagen, a biocompatible natural macromolecule, is able to support cell adhesion and proliferation due to its specific molecular structure and bioactivities. Moreover, collagen can be used as a kind of reservoir to carry bioactive molecules. Collagen-based vehicles have been used to deliver growth factors, such as acidic fibroblast growth factor (aFGF) and bFGF by association with heparin, or by means of a biotinylation process. Tissue regeneration and vascularization in vivo has been accelerated by collagen sponges incorporated bFGF-loaded microspheres. Collagen can be manufactured in various forms by varying the process, employing different freezing, drying and/or cross-linking process and, it has been shown that the differences in the collagen structure can influence the release of loaded substances. Rosenzweig et al. investigated the release properties of fluorescent drug analogs which bonded covalently with collagen, and found a clear correlation between the release profile of the model drug and the structure of the collagen gels. The relationship was further confirmed by Ungaro et al. who suggested that both microsphere and polymeric composite scaffolds affected the release rate of protein. Therefore, it was speculated that an anisotropic collagen matrix could provide a spatiotemporal-controlled release model of growth factor with lessening diffusion into surrounding uninjured areas during tissue regeneration.

In this study, we prepared a kind of inhomogeneous double-layered collagen membrane incorporating bFGF-loaded chitosan-heparin (CS-Hep) nanoparticles. The nanoparticles were prepared by a polyelectrolyte gelation process, and then were sandwiched between the double-layered collagen membranes. To achieve bFGF spatial controlled release from the membranes, one dense layer and one loose layer of collagen each having a different porosity were designed and prepared by employing different approaches. The dense and loose layers of collagen were combined to construct the double-layered membrane. In addition, human serum albumin (HSA) was chosen as a kind of model protein and its release profiles from the double-layered membrane were investigated and, finally, the bioactivity of the growth factor on fibroblast cell (L929) was evaluated in vitro.

MATERIALS AND METHODS

Preparation of CS-Hep nanoparticles

Chitosan (CS, M_w = 100 kDa, 90% deacetylation)-heparin (Hep, sodium salt, 150 μg/mg) nanoparticles (CS-Hep nanoparticles) were prepared using the polyelectrolyte gelation process. Briefly, 2 mL of acetic acid solution of CS (pH 6.0, 2 mg/mL) was dropped slowly into 1 mL of aqueous solution of Hep (5 mg/mL), and stirred at 700 rpm in an ice bath. The resulting CS-Hep nanoparticles were characterized by transmission electron microscope (TEM, JEM-100C XII), dynamic light scattering (DLS, MALVERN, Nano-ZS) and infrared spectra (IR, BIO-RAD, FTS300MX), respectively. To prepare recombinant human bFGF (rhbFGF)- or HSA (Sigma)-loaded nanoparticles, a calculated amount of rhbFGF or HSA was mixed with the Hep solution overnight at 4°C and then the above process was employed to fabricate bFGF- or HAS-loaded nanoparticles. The nanoparticles were stored as suspension for future use.

Preparation of collagen layers

Native insoluble type I collagen was isolated from bovine Achilles tendon by means of enzyme digestion in our laboratory. Typically, 0.6% (w/v) collagen solution was poured into a stainless steel tray and then freeze-dried to create a collagen sponge, which was then compressed between poly(tetrafluoroethylene) (PTFE) plates overnight. Similarly, 0.6% (w/v) collagen solution was poured into a stainless steel tray in a clean bench at room temperature, and another kind of collagen thin film was fabricated by means of air-drying. For the cross-linking of the collagen membranes, a solution was prepared using D-ribose (4% w/w), aceticone (10% w/w), and Milli-Q water; and the pH was adjusted to 8 by ammonia. Both the collagen sponge and film were immersed into the cross-linking solution for 48 h. The cross-linked materials were then washed carefully and soaked in ultra-pure water overnight and then freeze-dried or air-dried again. The air-dried layer was used as the dense layer, and the freeze-dried one as the loose layer. Then the collagen layers were characterized by scanning electron microscope (SEM, JSM-6700), atomic force microscope (AFM, multimode V) with tapping mode and IR.
Incorporation of bFGF nanoparticles into the double-layered collagen membrane

The suspension of nanoparticles was dropped slowly on the surface of the loose collagen layer which had been wetted and laid in a flat plate. The amount of bFGF was 100 ng/cm² of the membrane. The loose layer was then air-dried at 4°C, and the wet dense collagen layer was laid carefully on, followed by air-drying completely at 4°C. Thus, the double-layered collagen membrane incorporating bFGF-loaded nanoparticles was achieved. A blank membrane, without bFGF, was prepared by the same process. Both membranes were then examined by SEM.

Continuous flow apparatus construction

To test the spatial and temporal release properties of the membranes, a simple continuous flow apparatus (Fig. 1) was constructed with a constant flow pump (HL-2B, a digital constant flow pump, China), a silicone tube (diameter 5 mm), and a filter (Millipore). This continuous flow apparatus is easily adjustable to control the inflow velocity and to collect the outflow solution. The effects of differential structures on release kinetics could be detected, when layered materials with a diameter the same as that of the filter were put into the filter.

In vitro release kinetics of HSA from the double-layered collagen membrane

125I radio-labeled HSA (Na125I, PerkinElmer) was used as the model protein to assay the release kinetics of bFGF from the double-layered collagen membrane. 125I-HSA loaded CS-Hep nanoparticles were incorporated into the double-layered collagen membrane using the methods described previously. The simple continuous flow apparatus, as shown in Figure 1, was used to test the spatial and temporal releasing properties of HSA. The test sample (a wafer cut at a diameter of 1.3 cm) was put into the filter (diameter 1.3 cm) with the dense layer set as the input side and the loose layer as the output side, or reversed, as illustrated on the right side of Figure 1. Then the release medium (phosphate buffer solution, PBS, pH 7.4 or 5 mg/mL collagenase solution in PBS) was pumped at a constant velocity at 37°C, under static condition. At preset time intervals, the outflow solution was collected for 20 days (10 days for collagenase solution), and the inflow solution was replaced with the fresh release medium at the same time. The collected solution was stored at −20°C, and then its radioactivity was determined using a liquid scintillation counter (Gamma Radioimmunoassay Counter SN-6100, Shanghai Hesuo Rihuan Photoelectric Instrument CO. LTD China). The experiments were performed in triplicate. The total radioactivity of the membranes was calculated by determining the radioactivity of small pieces of dry membrane with a mean weight of ca. 0.48 g. The cumulative release percentage was calculated as a function of time. To make the release course visible, fluorescein-isothiocyanate (FITC) labeled HSA was used instead of 125I radio-labeled HSA, which was synthesized as reported previously. The membranes with FITC-labeled HSA were immersed in PBS for 1 h, and then were taken out and air-dried (the thickness of the wet membrane increased from 300 μm to more than 2 mm and influenced the transmission of light). The dried membranes were put on the glass-bottomed dish which was set on a confocal laser scanning microscope (CLSM, LSM710, Carl Zeiss, Jena, Germany). A three-dimensional (3D) image of the center of the membrane was scanned along with the longitudinal membrane depth from base surface (Z) with Z axial interval 20 μm. Then the same membranes were immersed in PBS for another 6 h, and treated and examined as described above. The signal intensities of fluorescence were obtained by excitation at the wavelengths of 488 nm.

In vitro cell culture and proliferation assay

Both the double-layered collagen membranes with and without bFGF were cut into wafers with the diameter of ca. 1.5 cm and placed into 24-well cell culture plates. After sterilization using Gamma irradiation, the wafers were then immersed in 1 mL of fresh cell culture medium, RPMI-1640, for 12 h. After 12 h, the cell culture medium was suctioned out, and fibroblasts L929 were seeded on the surfaces of the loose layer or the dense layer of the double-layered collagen membrane with and without bFGF at a cell density of 1 × 10⁵ cells/membrane and incubated at 37°C in 5% CO₂ for 5 h. The membranes were then placed in new culture plates to remove any cells which had not adhered well to the surfaces of membranes. RPMI-1640 were then added to each well and cultured for 3 or 5 days. The culture medium was changed every 2 days. At the third and fifth day, the cultured cells on the membranes were harvested by trypsin...
digesting to avoid the membranes interfering with UV absorption in the process of tetrazolium salt assay. Subsequently, the cells were incubated for 3 h with tetrazolium salt [MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2,5-tetrazolium bromide, Gibco, final concentration 0.5 mg/mL] at 37°C. The formed formazan crystals were solubilized and the absorbance was measured at 490 nm and 630 nm (as background) wavelength by a microtitrator plate reader (Thermo Varioskan Flash 3001). The freeze-dried collagen sponge was set up as the control group, using the average absorbance of the third day’s control group as the base to calculate the cell’s adhesion and growth rates. Some of the double-layered collagen membranes with bFGF and cells cultured for 3 days and 5 days period were fixed by 4% paraformaldehyde in PBS at room temperature. After rinsing three times with PBS, the fixed specimens were subjected to cell nucleus staining with 0.05% propidium iodide (PI, Molecular Probes) for 45 min and then to washing with PBS. The stained specimens were put on the glass-bottomed dish set on the CLSM. 3D images were scanned along with longitudinal gel depth from base surface (Z) with Z axial interval of 11.80 μm. The signal intensities of fluorescence for the nuclei were obtained by excitation at the wavelengths of 488 nm.

Statistical analysis
The results of all experiments were presented as the mean values ± standard deviation (SD). Comparisons between the two HSA release groups were performed with a two-tailed Student’s t-test and differences between the two datasets were considered significant when p < 0.05. All cell proliferation data analyses were performed using OriginPro 8 with variance analysis (ANOVA) and Holm-Bonferonni tests for differences among the groups. Values of p < 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION
Characterization of the CS-Hep nanoparticles
Because CS and Hep have many advantages for applications in the biomedical field, including good biocompatibility, suitability for protein delivery,34,35 and ease of fabrication,30,31 they were chosen to prepare the nanoparticles to carry the bioactive molecules in this study. Figure 2(a) shows the typical TEM image of the CS-Hep nanoparticles with irregular global shape. The average diameter determined by DLS was 149.5 nm [Fig. 2(b)], which was in accordance with the size in the TEM image. The value of zeta potential was −38.9 [Fig. 2(c)], which indicated that the negatively charged Hep was enriched on the surface of the CS-Hep nanoparticles. Figure 2(d) presents the IR spectra of CS, Hep, and CS-Hep nanoparticles. Compared with the IR spectrum of CS, it could be seen that the amino absorption value (1580–1630 cm⁻¹) narrowed to 1630 cm⁻¹ and the peak at 1226 cm⁻¹ appeared in the spectrum of the CS-Hep nanoparticles. We believe these IR spectrum changes occurred due to the formation of complexes between the −NH₂ groups of chitosan and the carboxyl and sulfonic groups of heparin by electrostatic forces.36,37 The major IR properties of the CS-Hep nanoparticle mainly consisted of the IR spectrum of Hep because the molecular weight of Hep was smaller than CS, which indicated that more Hep molecules existed in the nanoparticles and which was consistent with negative zeta potential charge. We supposed that the CS-Hep nanoparticles might tend to incorporate bFGF due to the specific binding affinity of Hep with a number of biologically important proteins, such as growth factors and cytokines, which is helpful in protecting bFGF from denaturation and enzymatic degradation.38–41 Moreover, the negatively charged surface of CS-Hep nanoparticles can interact with collagen by electrostatic interaction,35–40,42 which is useful for the adsorption of the nanoparticles on the collagen membrane.

Characterization of collagen layers
Figure 3(a,b) shows the typical SEM images of the freeze-dried collagen sponge and the air-dried collagen film, respectively. Open and interconnected pores were observed in the surface of the collagen sponge; whereas a dense flat surface without obvious pores was formed on the collagen film. It should be noted that the collagen fibers on both the sponge and collagen film maintained the specific D-period structure [Fig. 3(c,d)]. In addition, we investigated the effect of D-ribose on the collagen membrane by IR spectroscopy. As shown in Figure 3(e,f), it can be observed that several changes occurred in the spectrum of the D-ribose cross-linked collagen film in comparison with that of the uncross-linked film. The characteristic bands of uncross-linked collagen film that located at 1628, 1552 and 1234 cm⁻¹ (amide I, II, and III) [Fig. 3(f)] shifted to 1633, 1551, and 1204 cm⁻¹ after cross-linking, respectively, and a new characteristic band in the range of 900–1100 cm⁻¹ appeared. These recorded events indicated that the collagen was cross-linked by D-ribose through nonenzymatic glycation.43 The characterizations of collagen sponge and film implied that neither freeze-drying nor air-drying influenced the 3D structure of the collagen fibers and both methods could be used for manufacturing collagen materials.

Characterization of the double-layered collagen membrane
It is clear in Figure 4(a) that the double-layered collagen membrane consisted of a dense layer and a loose layer. The outer surface of the loose layer was made tighter than it was originally, but the whole structure was less compacted than the dense layer as shown in Figure 4(a,b). It can be observed that the CS-Hep irregular global nanoparticles aligned on the inner surface of the loose layer [Fig. 4(c)]; this effect is ascribed to the electrostatic interaction between the negative charge of Hep and the positive charge of collagen.30,42 It is worth mentioning that the bFGF loaded nanoparticles were used as the suspension solution in this study. That is to say, both the free bFGF and encapsulated bFGF were absorbed on the inner surface of the loose layer. The multiform of bFGF would enhance the efficiency of the protein and vary release profile in comparison with previous traditional incorporation methods.13
Release of HSA from the double-layered collagen membrane

Figure 5(a,b) presents the release profiles of the model protein HSA via detecting radio activities of the collected media. To investigate the effect of the structure of the double-layered collagen membrane on the release profile of HSA, two flow directions were employed: the dense layer to the loose layer and the loose layer to the dense layer. When PBS was used as the release medium, the release profiles of HSA in both the two flow directions revealed that they were in a biphasic pattern, no matter which layer of the membrane was the output side or input side. It was found that the sustained release followed the obvious initial burst release after the first 12 h. Nevertheless, the average release rate of HSA from the loose layer to the dense layer was slower than that of the dense layer to the loose layer after burst release. The cumulative release of HSA from the loose layer to the dense layer was 56.39% in 20 days, while HSA released from the dense layer to the loose layer reached 69.39% [Fig. 5(a)]. It appears that diffusion was the main factor influencing the sustained release profiles in PBS, although the burst release resulted from the side leakage. From the dense layer to the loose layer, diffusion of HSA driven from high resistance to low resistance induced fast release. Conversely, diffusion from the loose layer to the dense layer was slower. The initial burst release and the early release rates of HSA in the collagenase solution were slower than those in PBS [Fig. 5(b)], which was due to the deposition of collagenase on the membranes as a kind of biomacromolecular partial obstruction of the flow. Subsequently, the HSA release rate increased as the membranes were digested by collagenase. The membranes remained as gels at 1/3–1/2 of their original size without being completely digested at the 10th day, which means the release was controlled by the digestion of the membranes after the burst release. The release of HSA within the collagenase solution flowing from the loose layer to the dense layer was faster than that from the dense layer to the loose layer, which was due to the collagenase digestion rate for the loose layer was faster than that for the dense layer due to their different structures as was described in a previous report.\(^\text{44}\) We considered that the release from the dense

FIGURE 2. TEM image (a), size distribution (b), and zeta potential (c) of CS-Hep nanoparticles. (d) IR spectra of CS, Hep, and CS-Hep nanoparticles.
FIGURE 3. SEM images of the loose layer (a, bar 100 μm), the dense layer (b, bar 10 μm); (c) SEM image of the loose layer with higher magnification (bar 1 μm); (d), AFM image of the dense layer (scale 3.8 μm). IR spectra of the cross-linked dense layer (e) and the uncross-linked dense layer (f).

FIGURE 4. SEM images of the double-layered collagen membrane. (a) the cross-section view; (b) the outer surface (bar 100 μm); and (c) inner surface with nanoparticles on the loose layer (bar 100 nm).
layer to the loose layer in the collagenase solution was controlled by diffusion, but side leakage was the main factor inducing the faster release rate from the loose layer to the dense layer in the double-layered collagen membrane due to the difference of enzyme resistance. In Figure 5(c), the 3D CLSM images of the membranes incorporated with FITC-labeled HSA are presented. The FITC-labeled HSA diffused through the double-layered collagen membrane and the fluorescence intensities decreased over time. It was found that most of the FITC-labeled HSA moved from 50 to 150 μm at the first hour to 150 to 200 μm or higher at the seventh hour whereas a few of HSA lasted less than ca. 100 μm as shown between the double lines in Figure 5(c). This indicates that the FITC-labeled HSA diffused more easily to the loose layer than to the dense layer at the center of the membrane. The different release rates from the different exposed layers of the double-layered membrane in the solutions reveals that the release profiles were in a spatiotemporal model, which displays a clear correlation with the structures of collagen membranes.

For growth factors delivery, their appropriate tissue exposure in a spatiotemporal role is required. Moreover,
is also critical to avoid undesirable side-effects due to the undesired release to the surrounding site.7,9 Such release to the peripheral tissue may induce unpredictable outcomes, such as hypertrophy (increase cell size), hyperplasia (increase cell number), metaplasia (change cell type), unwanted vessel growth, or ectopic bone formation.3,45 Most developed growth factor delivery systems can sustain the release of the proteins for a long period, especially scaffolds incorporating growth factor loaded microparticles or nanoparticles.13–15 In the present study, the model protein HSA demonstrated the capability to sustain release in the delivery system over 20 days in PBS and for more than 10 days in the collagenase solution. The release time was a relatively long period to match the long timeframes of tissue regeneration, which range from days to weeks.13 It was further demonstrated that the as-prepared inhomogeneous layered collagen membranes showed different protein releasing profiles with different flow direction of release medium, which confirmed that the structures of scaffolds influence the sustained release rate. The most important result was that the smaller release volume in one direction and the greater release volume in another direction suggested that the inhomogeneous membrane could reduce the undesired release to the surrounding site, as is illustrated in Figure 6. In theory, the protein loaded in the inhomogeneous double-layered collagen membrane could be released with more precise structure-controlled orientation than that released from a homogeneous implant. Therefore, the constructed inhomogeneous layered collagen membrane incorporating growth factor-loaded nanoparticles could provide a spatio-temporal control over the growth factor delivery system and could target a specific site while lessening undesired release.

Cells adhesion and proliferation
To reveal the effects of bFGF activities and structures of the double-layered collagen membrane on cell adhesion and proliferation, L929 cells were seeded directly onto the surface of the membranes. Figure 7(a) shows the 3D CLSM images of the cultured cells adhering to the membranes, and it was found that the cells infiltrated deeper and with higher intensities on the loose layer of the double-layered membrane with bFGF than did of the cells on the dense layer of the membrane. As was expected, the cells’ fluorescence intensities increased from the third day to the fifth day, and the fluorescence intensity of cells cultured on the dense layer of the membrane was weaker than that of the cells on the loose layer at both times. Figure 7(b,c) shows the results of cell adhesion and proliferation. The L929 cells proliferated on all membranes and all the cell numbers increased from the third day to the fifth day. Figure 7(b) shows that both membranes without bFGF could support cell adhesion and growth, but the average numbers of cells on both the loose layer and the dense layer of the double-
layered collagen membrane without bFGF were less than the number on the control freeze-dried collagen sponge, especially at the fifth day. The result can be explained by the inhomogeneity of the structure of the double-layered membrane. In particular, the dense layer may influence the transport of the culture medium. With the incorporated bFGF, the average numbers of cells on both the loose layer and the dense layer were greater than that of cells on the control collagen sponge. It is interesting to find that there was no significant difference between the number of cells on the dense layer of the membrane with bFGF and on the control sponge, but significant difference presented between the number of cells on the loose layer of the membrane with bFGF and on the control sponge. These results imply that L929 cell adhesion and proliferation were affected not only by bioactive bFGF but also by the spatial microstructure of the supporting matrix. Furthermore, Figure 7(c) shows that both layers of the double-layered membranes with bFGF displayed higher cell growth rate than did the membranes without bFGF at the third and the fifth day. It was confirmed by variance analysis (ANOVA) that this significant difference was induced by the stimulating effect of bFGF. The number of cells on the loose layer of the membrane with bFGF was much greater than that of cells on the dense layer of the membrane at the third day ($p = 0.011 < 0.05$) and the fifth day ($p = 0.015 < 0.05$), which was consistent with the above results when both these two groups were compared with the control group. Therefore, this study confirms that the loose layer of the double-layered membrane with bFGF would be the best substrate for L929 growth.

Collagen is able to support various kinds of cell adhesion and proliferation due to its specific bioactivities via its interaction with integrin, and it has been used widely for tissue regeneration.\(^\text{18-22}\) In the present study, all the collagen membranes were proven to support L929 cell adhesion and growth, which was attributed to the maintenance of the 3D structure of the collagen fibers that could interact with the cells. However, the inhomogeneous membranes without bFGF induced less cell growth which indicated that the structure of the membranes did affect the cell proliferation. Previous reports revealed that different surface properties such as pore size and porosity would result in different rates of cell growth.\(^\text{16}\) In this study, the different cell growth rates resulted from the inhomogeneous structures that influenced the culture medium transport. Because of the stimulation of bFGF, the cells grew faster on the membrane incorporating bFGF than they did on that without bFGF. These results revealed that the bFGF maintained the bioactivities during the process. It should be noted that the growth rate of cells on the dense layer of the membrane with bFGF was the same as that of the cells on the control sponge, but cells on the loose layer of the double-layered membrane with bFGF had a higher growth rate than did cells on the control sponge. Although the structure could induce different cell growth rates, there was no significant difference of cell growth rate between the cells grown on the dense layer and those grown on the loose layer of the double-layered membrane without bFGF, which means that the significant difference of cell growth rate between the cells grown on the loose layer and on the dense layer of the double-layered membrane with bFGF would be a result of the different amount of the released bFGF. Statistical analysis confirmed that the bFGF was the main reason for the significant difference. Combining the cell assay results with the HSA release results in PBS, it was determined that the different diffusion gradients of bFGF induced different cell growth rates. Therefore it was anticipated that the bFGF could be efficiently released in desired sites when implanted locally, filling the wound, while decreasing the undesired spreading to surrounding tissues, as Figure 6 illustrates, to avoid side effects such as scar formation.\(^\text{17}\) The spatiotemporal control over growth factor delivery did induce different biological functions, which were consistent with previous reports.\(^\text{45}\) Continuous gradients of chemical signals such as growth factors are known to induce concentration dependent cell type-specific responses.\(^\text{46-48}\)

Recently, the Mooney group constructed a kind of double-layered scaffold loaded with a lower amount of vascular endothelial growth factor (VEGF) in layer 1 and a higher amount in layer 2. This differential loading formed a spatial concentration gradient, and the spatial VEGF delivery resulted in faster restoration of hind limb blood flow to normal levels than did by VEGF delivery without spatial control.\(^\text{1}\) In our study, the constructed double-layered collagen membrane sustained release of the protein spatially and induced significant difference in cell proliferation when the cells were seeded on the different layers of membrane with bFGF in vitro. It provided an alternative spatiotemporal delivery system, while lowering undesired spreading to surrounding, uninjured areas and could have potential applications in the field of tissue regeneration.

CONCLUSIONS

In summary, an anisotropic double-layered collagen membrane incorporating bFGF-loaded CS-Hep nanoparticles between the double layers was prepared. The protein release was observed in a spatiotemporal control model from the collagen membrane by release assay in a continuous flow apparatus and cell proliferation assay in vitro. The bFGF released from the membranes maintained the bioactivity due to the mild process conditions and stimulated the cell proliferation. The as-prepared double-layered collagen membrane with bFGF has promising applications for growth factor delivery for tissue repair.

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