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Heparin-functionalized collagen matrices with controlled release of basic fibroblast growth factor

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Abstract Tissue engineering scaffolds with controlled long-term release of growth factors are constructed in an attempt to mimic the intelligent ability of the extracellular matrix (ECM) to release endogenous growth factors. In this study, collagen sponges (Collagen group) were modified by *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) crosslinking (EDC/NHS group) and heparin immobilization (EDC/NHS-H group), and subsequently seeded with human umbilical vein endothelial cells (HUVECs). Native and modified sponges were pre-adsorbed with basic fibroblast growth factor (bFGF) to evaluate the sustained release and bioactive maintenance of bFGF from the sponges. We found that modified collagen matrices permitted HUVECs to proliferate and migrate well and to distribute uniformly. The EDC/NHS-H group exhibited an excellent sustained-release profile and bioactive maintenance of the pre-adsorbed bFGF as compared with the Collagen and EDC/NHS groups. These results suggest that heparin-functionalized collagen matrices can support a controlled release of bFGF and thus, have potential as a tissue engineering scaffold.

Keywords Collagen matrix · Basic fibroblast growth factor · Heparin · Crosslinking · Controlled release

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1 Introduction

Biodegradable scaffolds play an important role in tissue engineering by providing mechanical and biofunctional supports for both differentiated and progenitor cells. An ideal scaffold includes a three-dimensional porous construct that can approximate the structure and biological function of the native extracellular matrix (ECM), in which cells proliferate, differentiate and migrate [1]. The cell–cell and cell–ECM signaling is mediated by growth factors, which are involved in the dynamic properties of ECM via specific cell-surface receptors [2, 3].

The activities of heparin-binding growth factors, such as basic fibroblast growth factor (bFGF), are primarily maintained by heparan sulfate proteoglycans contained within the ECM [4, 5]. The control and release of bFGF, which is known to promote angiogenesis and proliferation of mesenchymal cells, through interactions with ECM–heparan sulfate represents a prototype of such a system [6–8]. On the other hand, fibrillar collagens like type I collagen, the major constituent of the ECM, are known to possess only a low affinity for growth factors [9]. In particular, heparin-functionalized biomaterials have been used in protein delivery owing to the importance of their highly anionic characteristics [10]. Heparin has the highest electronegative charge of any known biological molecule, which mediates its important biological role as a multivalent binding agent for many proteins, including growth

factors, to protect against degradation and potentiate receptor binding [11, 12]. Accordingly, heparin has been incorporated covalently into numerous drug delivery vehicles with a controlled release of growth factors [13–16].

Mimicking the structure and function of the ECM, the intelligent integration of collagen, heparin and growth factors (such as bFGF) will determine the success of tissue engineering scaffolds [17, 18]. Although, collagen cannot be directly used as a tissue engineering scaffold due to its poor mechanical strength and fast rate of biodegradation [19]. These properties can be improved, however, by chemical crosslinking methods. Collagen matrices crosslinked by *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) are reported to be non-cytotoxic in vitro and exhibit good biocompatibility in animal studies, whereas other crosslinking agents, such as aldehydes and epoxide, are known to induce cytotoxic reactions in vitro or in vivo and hamper endothelialization [20–22].

Here, our aim was to investigate a simple and novel method for fabricating three-dimensional scaffolds similar to the ECM in structure and function. Collagen sponges were modified by EDC/NHS crosslinking, or by EDC/NHS crosslinking and heparin immobilization, under a one-step simultaneous procedure. In this work, the focus was on the sustained-release performance and bioactive maintenance of bFGF in vitro after the collagen matrices were preabsorbed with bFGF, in addition to evaluations on morphological and biological behaviors.

2 Materials and methods

2.1 Growth factors and reagents

Recombinant human bFGF was purchased from Shuanglu Biological & Pharmaceutical Ltd Co (Beijing, China). The standard solution of bFGF was provided by China Institute of Drugs and Bioproducts (Beijing, China). Heparin sodium salt (grade I-A from porcine intestinal mucosa) was purchased from Huiguang Co (Shanghai, China). EDC, NHS, bovine serum albumin (BSA) and an enzyme-linked immunosorbent assay kit (ELISA) were obtained from Sigma-Aldrich (St Louis, MO). Deionized water (18 MΩ) was used in all experiments (RMLD's EDI-ultrapure water systems, Tianjin, China). All of the chemicals were reagent grade.

2.2 Fabrication of collagen sponge

Finely filtered and homogenized collagen was obtained from porcine skin after alkaline or acidic swelling, or by a combination of both procedures [23, 24]. The composition and purity of the extracted collagen was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and amino acid analysis. The collagen was dissolved at 15–25 mg/ml in 0.1% acetic acid solution and homogenized in a Waring Blender. The mixture was frozen in a culture disk at a temperature of −20 to −50°C. The frozen mixture was lyophilized to produce a collagen sponge.

2.3 Crosslinking and heparinization of collagen sponges

Each collagen sponge was crosslinked using EDC and NHS with or without heparin. Briefly, the dried collagen sponge was incubated in 0.05 M buffer of 2-morpholinoethane sulfonic acid (MES, pH 5.60) for at least 30 min, and then immersed in a solution of EDC and NHS in MES buffer [25]. The crosslinking reaction was allowed to proceed for 4 h at 37°C under gentle shaking. Then, the matrix was washed with 0.1 M Na_2HPO_4 solution (2 h), 4 M NaCl (four times in 24 h) and deionized water (five times in 24 h), respectively. For heparin-containing sponges, heparin was added into the MES buffer including EDC/NHS and activated for 10 min. Approximately 30 mg heparin was incorporated per gram of collagen sponge by a colorimetric method [26]. After the modification procedure, matrices were frozen at −80°C overnight and lyophilized for 36 h. Matrices treated with EDC/NHS in the absence of heparin were designated as the EDC/NHS group and those including heparin were designated as the EDC/NHS-H group, while the native collagen sponge as the Collagen group.

2.4 Determination of primary amino groups

The residual amounts of free primary amino groups in the collagen matrices before and after modification treatment was determined using 2,4,6-trinitro benzene sulfonic acid (TNBS), according to previously described methods [27, 28]. Briefly, 5-mg piece of the collagen matrices was placed in a flask with 1 ml $NaHCO_3$ solution (4%, w/v) and kept at room temperature for 30 min. One ml TNBS solution (0.5%, w/v) was then added and incubated at 40°C for 2 h. After 3 ml 6 M HCl solution was again added, the mixture was allowed to hydrolyze at 60°C for 90 min. The obtained mixture was diluted with 5 ml deionized water and cooled to room temperature. Finally, the absorbance was measured at 420 nm. Glycine was used as control to make the standard curve. The experiments were performed in triplicate.

2.5 Morphological observation

Collagen matrices (about 5 mm × 5 mm × 5 mm) were quickly taken out and cut into thin pieces (about 1 mm in thickness) by a keen scalpel after immersed in the liquid nitrogen for 10 min, and then observed using a scanning electron microscope (SEM, JSM 6700F NT) after coated gold.

2.6 Cell culture and assays

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. One ml of the obtained HUVEC suspensions with cell densities of 300 × 10^4 cells/ml was seeded into each group of collagen matrices (5 mm × 5 mm × 5 mm) in tissue culture polystyrene (TCPS) flasks (Costar, NY, USA) and cultured statically for approximately 2 weeks in R1640 media (Gibco) containing 10% FBS (Sijiqing Bioeng Co, Hangzhou, China), 100 U/ml penicillin and 100 μg/ml streptomycin. The culture medium was refreshed every day. HUVECs in different groups were observed under a confocal laser scanning microscope (CLSM, Bio-Rad Radiance 2100), after fluorescein diacetate (FDA, Aldrich) staining [29].

To analyze cell viability, pieces (5 mm × 5 mm × 5 mm) of collagen matrices in different groups were incubated in R1640 media without FBS at 37°C for 3 h, and then placed in 24-well TCPS plates. One ml of HUVEC suspensions (4 × 10^4 cells/ml) was seeded on each piece and cultured statically in R1640 containing 10% FBS for 7 days [30]. Culture medium was replaced every 2 days. On days 2, 4 and 7, cell viability was measured via MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay by the absorbance at 490 nm. The experiments were performed in quintuplicate.

2.7 In vitro absorption of bFGF to collagen matrices

For in vitro absorption of bFGF, 5 mg collagen sponge sheets of both Collagen and EDC/NHS groups were incubated with 0.5 ml bFGF solution (1 ng/ml) in PBS (0.14 M NaCl, pH 7.2) containing 1 mg/ml BSA, for 72 h at 37°C under gentle shaking. The EDC/NHS-H group sponge sheets were incubated with PBS (0.6 M NaCl, pH 7.2) containing 1 mg/ml BSA under the same conditions. Thereafter, the samples were washed in 1 ml PBS (twice for 5 min) to remove all non-bound bFGF and kept in 4°C for further uses [31].

2.8 In vitro release experiment

Collagen matrices pre-absorbed with bFGF were placed in a plastic tube containing 1 ml of the release medium (PBS containing antibiotics, pH 7.2) under gently shaking at 37°C. The concentration of released bFGF in the medium was evaluated over time using an ELISA assay. The medium was exchanged with fresh medium after each measurement on days 0.5, 1, 2, 5, 8, 11, 14, 17, 21, 25, 29, 33 and 37. A calibration curve of standard bFGF solutions via the absorbance at 492 nm was constructed using an ELISA plate reader. Each experiment was performed in quintuplicate.

2.9 Assay of the activity of bFGF-loaded matrices stored at 4°C

The bFGF-loaded matrices stored at 4°C were evaluated on days 2, 4, 11, 13, 17, 21, 26, 31, 36, 41, 46 and 57, and restored at −70°C for the subsequent MTT assay. The activity was tested according to the previous method [32–34]. Each test was performed in quintuplicate.

2.10 Statistical analysis

The data are presented as the mean ± standard deviation. Statistical analysis and simple regression analysis were performed using SPSS11.0, and P-values of <0.05 and <0.01 were considered statistically significant.

3 Results

3.1 Residual amounts of free primary amino groups

Upon crosslinking with EDC/NHS and heparin immobilization, the number of free primary amino groups in the collagen matrices decreased as shown in Fig. 1. Crosslinking and heparin immobilization resulted in that the number of free primary amino groups in EDC/NHS and EDC/NHS-H groups was approximately 230.2 and

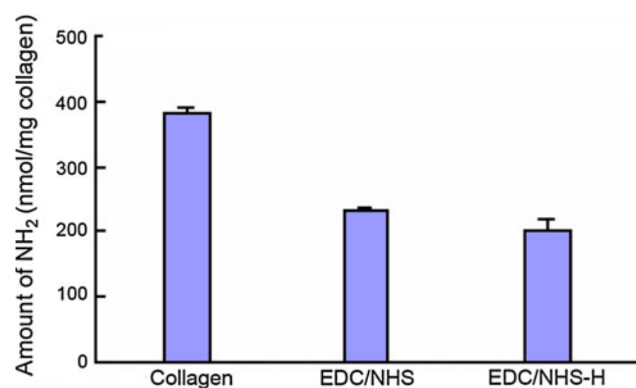

Fig. 1 Residual amounts of free primary amino groups. The values represent the mean ± standard deviation ($n = 3$). There was a significant difference among the three groups ($P < 0.05$)

203.5 nmol/mg, respectively, changing from 381.3 nmol/mg of the Collagen group. There were significant differences between the Collagen and the EDC/NHS groups ($P < 0.01$), as well as between the Collagen and EDC/NHS-H groups ($P < 0.01$).

3.2 Morphological characteristics

The morphology of collagen matrices was analyzed by SEM. From the SEM images shown in Fig. 2a–c, no significant differences among the three groups were observed. The shape of the pore in three groups was irregularly round. The pore diameter was approximately 120–260 μm in the Collagen group. EDC/NHS crosslinking resulted in a little increase to about 155–300 μm in pore diameter, and the diameter became 130–300 μm by further treatment of heparin immobilization.

3.3 Cell viability

The CLSM images of HUVECs growing within the three groups of the collagen matrices after 2 weeks of culture and FDA staining are also shown in Fig. 2. The growth of HUVECs on the surfaces of the EDC/NHS and EDC/NHS-H groups (Fig. 2e, f) was enhanced as compared to the Collagen group (Fig. 2d). Although HUVECs in the Collagen group were viable, there was less proliferation. In particular, cells on the EDC/NHS-H group proliferated well, coalesced and migrated from the wall to the inner pores of the sponge during the initial stages of culture, in contrast with that observed in the EDC/NHS and Collagen groups. Moreover, from the CLSM images, we observed that HUVECs in the EDC/NHS and EDC/NHS-H groups were distributed more uniformly and in a higher density than those in the Collagen group, in which many cells accumulated into aggregates. The porous structure of the Collagen group collapsed thoroughly, while it remained intact in the EDC/NHS and EDC/NHS-H groups.

Figure 3 demonstrates the MTT cell viability of HUVECs cultured within the different groups. The viable cell number of HUVECs in the EDC/NHS and EDC/NHS-H groups was enhanced compared to the Collagen group at days 2, 4 and 7 of culture. Furthermore, heparin immobilization appeared to enhance viable cell number at each time point. There were significant differences among both groups and time points ($P < 0.05$), which further confirms the CLSM results mentioned above.

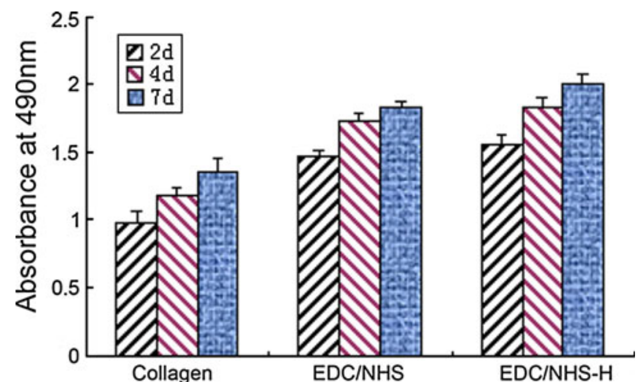

Fig. 3 Viability of HUVECs cultured in the three groups of collagen matrices. The data are shown as the mean ± standard deviation ($n = 5$). $P < 0.05$ among both groups and time points

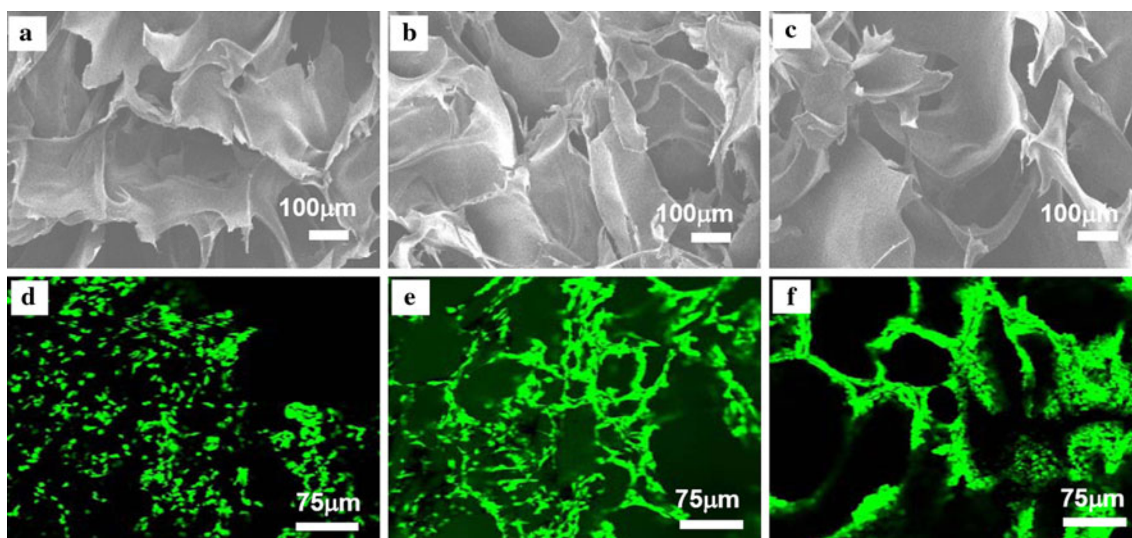

Fig. 2 SEM images (**a**–**c**) and CLSM images (**d**–**f**) of HUVECs in collagen matrices of the Collagen (**a**, **d**), EDC/NHS (**b**, **e**) and EDC/NHS-H groups (**c**, **f**)

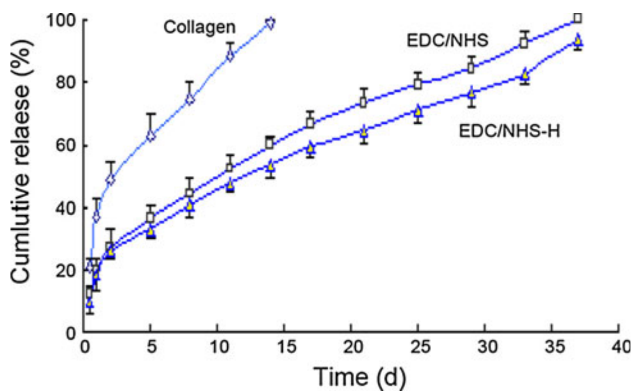

Fig. 4 Profiles of bFGF released from the three groups of collagen matrices. The values represent the mean ± standard deviation ($n = 5$). There was a significant difference between the EDC/NHS and EDC/NHS-H groups ($P < 0.05$ from days 14 to 37) and also between the Collagen and EDC/NHS groups ($P < 0.01$ from days 2 to 14)

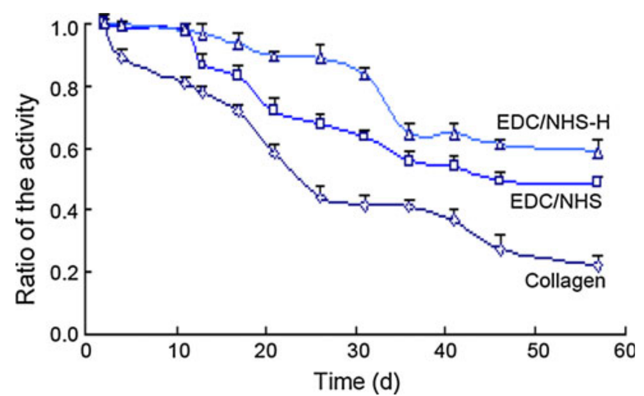

Fig. 5 In vitro bioactivity loss of bFGF-loaded collagen matrices stored at 4°C. The values represent the mean ± standard deviation ($n = 5$). There was a significant difference among the three groups ($P < 0.05$ from days 13 to 57)

3.4 Release kinetics of bFGF

The profile of bFGF release from the bFGF-collagen matrices was determined using an ELISA assay (Fig. 4). Pre-absorbed bFGF released from the Collagen group with an initial burst phase of 36.9% during the first 24 h and a subsequent rapid release. By day 14, 100% of the pre-absorbed bFGF had released from the Collagen group. Notably, the collagen matrix of the Collagen group had degraded into small debris by day 14. Contrarily, the EDC/NHS and EDC/NHS-H groups exhibited relatively small initial burst releases of 19.5 and 18.0%, respectively, and the release of bFGF from the EDC/NHS-H group was sustained over 37 days.

3.5 Activity of bFGF-loaded matrices over time

To determine any changes of bFGF activity after storage of bFGF-loaded collagen matrices at 4°C, we used an MTT assay to evaluate the ability of the complexes (bFGF-collagen matrices) to promote Balbc3T3 cell proliferation (Fig. 5). The loss of activity with time of the Collagen group pre-absorbed with bFGF solution was the most striking, while the bFGF–EDC/NHS-H group exhibited the least loss of activity. At day 57, only 21.9% of bFGF activity remained within the Collagen group, whereas 48.4% of the EDC/NHS group and 58.6% of the EDC/NHS-H group remained active.

4 Discussion

In order to develop tissue engineering scaffolds with controlled release of growth factors we used a system of collagen sponge, heparin and bFGF in this study. Collagen sponge was crosslinked using EDC and NHS, or using EDC, NHS and heparin under a one-step simultaneous procedure, resulting in a simple and novel method for fabricating three-dimensional tissue engineering scaffolds. In fact, collagen was considered to be a suitable tissue engineering scaffold in many reports [31, 35]. Collagen was crosslinked to stabilize the triple helix structure and immobilize heparin effectively, thus increasing the in vitro absorption of bFGF. The native collagen sponge (Collagen group) was crosslinked using EDC and NHS, which leads to formation of amide linkages between carboxylic acid groups and primary amino groups in amino acid residues of collagen [21]. Because of the consumption of amino groups, crosslinking of collagen results in the decrease of the free primary amino groups. The crosslinking degree, which is defined as the density of chemical junctions connecting the macromolecular chains to permanent network structure, was inversely proportional to the amounts of free amino groups [22].

The crosslinking degree was further increased by introduction of the activated heparin during the crosslinking process because the carboxylic acid groups in heparin also take part in crosslinking reaction with amino groups (Fig. 1). Collagen crosslinking and heparin immobilization were performed in a one-step simultaneous procedure, which is simple and efficient method for immobilization of heparin to collagen as compared with two successive procedures [36]. The heparin immobilization in the EDC/NHS-H group amounted to 30 mg (per gram of collagen matrix), which is higher than those using EDC or other carbodiimide, while heparin immobilization to non-crosslinked collagen is between 4 and 15 mg [37, 38]. This is the reason we used EDC in combination with NHS. Theoretically, the simultaneous procedure allows the reaction between the carboxylic acid groups of collagen or heparin

and free primary amino groups of collagen to take place randomly, thus causing the covalently bound heparin distributed quite uniformly across the collagen matrix. Contrarily, since the diffusion of heparin through the network structure of previously crosslinked collagen may become difficult, the two successive procedures of collagen crosslinking and subsequent heparin immobilization make a great probability that the covalently bound heparin are most located in the outer surface of collagen matrix, which may have a disadvantage when the covalently bound heparin are absorbed with bFGF to sustain and protect it.

It was found that the physicochemical properties of the resulting collagen matrices were improved greatly in our previous study. The shrinkage temperature, stability against collagenase, water-binding capacity, mechanical properties and morphological stability of the modified collagen matrices increased greatly. In this study, the scaffold morphology of the three collagen matrices, i.e., the Collagen, EDC/NHS and EDC/NHS-H groups, was not substantially altered by EDC/NHS crosslinking and heparin immobilization. The small changes of the SEM images in the pore diameter might result from the difference of the water-binding capacity in the three groups due to crosslinking degree. High scaffold porosity was deemed an attractive feature in that cell ingrowth across the matrices could readily be reached and mass transport and angiogenesis might be facilitated by such a highly porous structure. With average pore sizes larger than 120 μm, vascular ingrowth should be facilitated since pore sizes larger than 50 μm have been considered to be essential for in vivo angiogenesis [39].

Crosslinking usually makes the porous structure stable, so that the cells adhered and spread well in the EDC/NHS group, which is traits associated with good cell survival and biocompatibility [29, 40]. It was assumed that HUVECs excreted enzymes that digested the collagenous matrix after 2 weeks of culture. Additionally, the collagen fibrils may have enhanced the expression of collagenase genes in the HUVECs in vitro, thus degrading the collagen fibrils that were exposed on the surface of the Collagen group. However, after crosslinking treatment, the structure became stable and the surface smooth, and so there were few fibrils exposed. The further immobilization of heparin in collagen matrix not only degraded the matrix at a slower rate, but also approximated the components of ECM. Therefore, the cell proliferation and number in the EDC/NHS-H group was in the highest density (Figs. 2, 3).

Delivery systems that release bFGFs over a long period in a controlled manner may increase the efficacy of bFGFs for angiogenesis and tissue regeneration. The angiogenic effect of bFGF can stimulate vessel formation in vivo to improve the function of diseased tissue or increase the survival of transplanted cells by enhanced nutrient transport [41]. The initial burst releases of the three groups in this study differed greatly, with high one being not a good way for the bFGF release. As crosslinking degree increased, the number of the free primary amino groups of collagen matrix decreased as described above. Inversely, the residual and activated carboxylic acid groups of collagen matrix increased with EDC/NHS crosslinking and/or heparin immobilization. The previous study reported that ablation of carboxylic acid groups in gelatin, a denatured type I collagen, resulted in poorer interaction with bFGF, suggesting the importance of the negatively charged collagen side residues for interaction with bFGF [42]. Thus, it can be speculated that the positively charged heparin-binding domains in bFGF might play some role in the interaction with collagen matrix although any specific collagen binding sites in bFGF have not found presently. Therefore, the collagen sponge crosslinked by EDC and NHS, i.e., the DEC/NHS group, showed a lower burst release for bFGF. Of course, since the further heparin immobilization not only increased the carboxylic acid groups and introduced heparin into collagen matrix as well, the initial burst release for bFGF became the lowest (Fig. 4). All these derive from the activated carboxylic acid groups of the collagen matrices owing to the different crosslinking degree as discussed above. Of note, bFGF was released from the Collagen group in close accordance with the degradation of the material, as it came from gelatin hydrogels, glutaraldehyde-treated collagen sponge and bladder acellular matrix [31]. This result may confirm the hypothesis that degradation-based release of growth factors is a general character of collagen-based materials. In the present study the release time of bFGF from the EDC/NHS and EDC/NHS-H groups was over 37 days, longer than that in many previous reports. All these are related to the one-step simultaneous crosslinking and heparin immobilization.

On the other hand, when the collagen matrices of this study are used as tissue engineering scaffolds or defect repair substitutes in the future, bFGF is an essential ingredient for angiogenesis. The complex containing bFGF is faced with a loss in bioactivity due to the in vitro storage. Thus, the bFGF activity in collagen matrices was comparably tested after stored at 4°C for a period of time in this study (Fig. 5). This experiment has not found in the previous literature. The experimental results indicate that collagen crosslinking and heparin immobilization may, to some extent, protect the growth factors from bioactivity loss when the complex is stored for a long time. Although the mechanisms of bioactive retention did not totally remain clear, the simultaneous procedure of this study, randomly distributing the covalently bound heparin across the collagen matrix, maybe benefited the in vitro bioactive stability of bFGF–EDC/NHS-H complex. However, the

bioactivity loss of the complex (even the EDC/NHS-H group with bFGF solution) does not meet the need of substantial products, since the bioactivity loss of commercial bFGF is less than 20% during its storage at 4°C for 1 year. Recently, encapsulation technology has been utilized by our research group. For example, bFGF was encapsulated in poly(L,D-lactic-co-glycolic acid) (PLGA) microspheres and then introduced into biodegradable scaffolds [43–45]. The outer PLGA is used as a physical barrier to protect bFGF from environmental effect on its conformation. In a word, although the results of this study made a satisfactory achievement toward collagen matrices as biodegradable scaffolds, further studies will need to be performed, including angiogenesis in vivo and inhibition of bioactive loss.

5 Conclusions

In the present study, crosslinking and heparin immobilization of collagen sponges were carried out in simultaneous procedures under the same reaction conditions. We found that the crosslinking degree of the EDC/NHS-H group was higher than the EDC/NHS group. This modification method may regulate the biological characteristics of collagen matrices, which can then be used for tissue engineering scaffolds or defect repair substitutes. HUVECs proliferated and migrated well and distributed uniformly within the EDC/NHS-H group, in contrast to those within the Collagen and EDC/NHS groups. Additionally, the collagen matrix of the EDC/NHS-H group exhibited excellent sustained release and bioactive maintenance of bFGF. These results indicate that EDC/NHS crosslinking, combined with heparin immobilization, may effectively release and retain the bioactivity of bFGF and thus, have potential as tissue engineering scaffolds.

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