Controlled Delivery of Basic Fibroblast Growth Factor from Heparin-Conjugated Poly(L-Lactide-Co-Glycolide) Microspheres and Modified Collagen

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Abstract: Controlled delivery of basic fibroblast growth factor (bFGF) for a long time could be used as an angiogenesis therapy. In this study, novel heparin-conjugated poly (L-lactide-co-glycolide) (PLGA) microspheres (HCPMs) were developed for long-term, zero-order delivery of bFGF. HCPMs were prepared using a coupling reaction between PLGA graft with Amino (PLGA-NH_2) microspheres and heparin in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide. Modified collagen (M-collagen) was crosslinked using N-(3-dimethylaminopropyl)-N1-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). The release of bFGF from HCPMs was sustained for 3 weeks. The bFGF release was increased to more than 4 weeks using a delivery system of HCPMs suspended in M-collagen. The release was nearly zero order. The bioactivity of bFGF released from HCPMs in M-collagen was assessed using human umbilical vein endothelial cell (HUVEC) culture. bFGF released from HCPMs in M-collagen exhibited HUVEC growth for 15 days, similar to that of cultures to which bFGF in free form was added daily, suggesting that the delivery system of HCPMs in M-collagen can release bFGF in a bioactive form for a long period. This study shows that a bFGF delivery system using HCPMs in M-collagen exhibits controllable, long-term, zero-order release of bFGF and potentiates the angiogenic efficancy of bFGF administration.

Keywords: basic Fibroblast Growth Factor; Heparin Immobilization; Modified Collagen; Microspheres; Poly(L-lactide-co-glycolide)

1. Introduction

bFGF mainly acts on the mesodermal tissue, neuroectodermal cell and the differentiation of cells. Mechanical properties of bone was importantly improved by the local administration of bFGF [1]. The angiogenesis in wound heals was accelerated by bFGF [2,3]. However, as a kind of basic polypeptides, bFGF is sensitive to heat and acid, diffuses quickly in vivo, and can be zymohydrolysised easily. And its half-life period is short. So its biological effect can not be brought into full play. Delivery systems that release bFGF over a long period in a controlled manner may increase the efficacy of bFGF for angiogenesis and tissue regeneration.

Methods to sustain the delivery of bFGF have been reported previously. The method of impregnation of bFGF in alginate beads has a problem, and this delivery system release was completed within only 3 days [4]. A bFGF-loaded hyaluronate–heparin-conjugated hydrogel delivery system showed no initial burst and provided long-term release, but it was difficult to control the release rate and period [5]. And bFGF-loaded M-collagen delivery system is controllable, however, bFGF release from the delivery system within 4 weeks [6].

In this study, whether bFGF release from M-collagen can be prolonged by HCPMs suspended in M-collagen was investigated, and whether the cell growth of sustained bFGF delivery is further enhanced compared to bFGF delivery using M-collagen was explored. The bioactivity of bFGF released from the delivery system was examined by measuring HUVEC growth in vitro in a medium containing the bFGF delivery system.

2. Materials and Methods

2.1. Preparation of heparin-conjugated PLGA microspheres

PLGA, maleic anhydride, benzoyl peroxide（BPO） and acetone were put into a three-neck flask, stirred with nitrogen incoming, heated to 100℃ progressively, and reacted to PLGA graft with Maleic Anhydride (MPLGA) for 24h. After the reaction, ethylenediamine was put into a three-neck flask and dropped to 5-10℃ in low temperature water bath. MPLGA dissolved in tetrahydrofuran were dropped slowly into ethylenediamine dissolved in tetrahydrofuran, before increasing to ambient temperature, it was stir constantly and incubated for 10min. Then put reaction product into tetrahydrofuran (THF), after that, dropped them slowly into excessive distilled

water and collected the surface film. Repeated it until the wash liquor was neural or weak acidic conditions. After that, the product of PLGA-NH_2 was vacuum lyophilized for 96h (Fig. 1). PLGA-NH_2 was determined by the ninhydrin colorimetric method [7,8].

PLGA-NH_2 microspheres were prepared using the oil/water emulsion and solvent evaporation–extraction method (Fig. 2). Product was treated with methanol, acetone and dichloromethane, then it was dropped into phosphate buffered saline (PBS) solution to phacoemusification for 5min and dropped into Polyvinyl Alcohol (PVA) and aluminium isopropylate and stired constantly for 4h. After overnight evaporation at room temperature, the microspheres were washed with distilled water, and lyophilized for 3 days after being collected by centrifugation. PLGA-NH_2, Heparin and Sodium Chloride (NaCl) were disolved in MES Buffer Solutions as soon as NHS and EDC were put into the buffer solution. Then accumulative material was filtered through a filter, PLGA-heparin microspheres were collected by centrifugation. Unreacted materials were removed with sonication in distilled water for three times, and then lyophilized for 2 days [9].

The amount of heparin conjugated to the nanosphere surface was determined with the toluidine blue method. The HCPMs were added to the toluidine blue solution, and the mixture was agitated with a vortex mixer. Normal hexane was added, and the mixture was shaken vigorously, so that the toluidine blue–heparin complex could be extracted into the normal-hexane layer. After removing the HCPMs complexes from solution, the unextracted toluidine blue remaining in the water phase was determined by absorption at 631 nm with a UV spectrometer.

2.2. Crosslinking of collagen and contents of covalently linked

Collagen was prepared from bovine fedon. Collagen was crosslinked using N-(3-dimethylaminopropyl)-N1-ethylcarbodiimide hydrochloride (EDC) and N- hydroxysuccinimide (NHS). Before crosslinking, collagen was washed with MES buffer. Then the collagen were dropped into a solution of EDC and NHS in MES buffer, and gently shaken. After that the collagen was washed with Na_2HPO_4 solution and distilled water [10,11]. Crosslinked collagen was equilibrated with MES-buffer. Carboxylic acid groups of heparin were activated by adding EDC and NHS to a solution of heparin in MES-buffer. After pre-activation, crosslinked collagen was added to EDC/NHS activated heparin solution, giving a molar excess of heparin to free collagen primary amino groups [12]. After the reaction, the heparinized collagen, designated as Collagen-Heparin, was washed with Na_2HPO_4 solution, NaCl solution and distilled water.

5mg M-collagen was immersed to Toluidine Blue solution, the capacity was reached 25ml and 50ml by adding separately to NaCl and n-hexane. Toluidine Blue-Heparin was separated from water by strenuous oscillation in 1min. water was diluted by 1:10 of anhydrous ethanol, and the absorbance was detected with spectrophotometer. The contents of heparin were calculated by the standard curve.

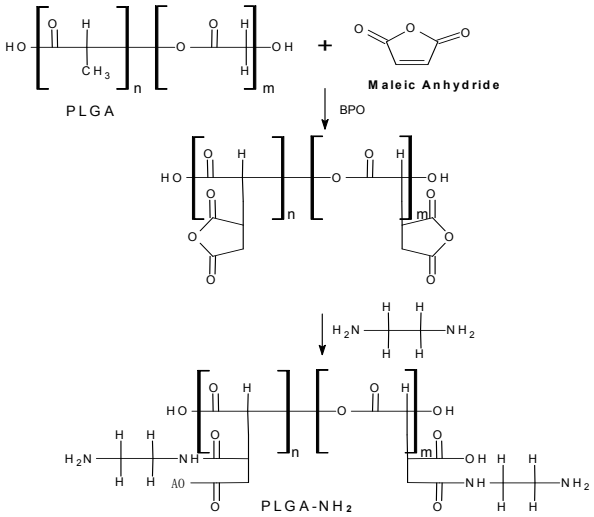

Fig.1. Reaction sequence for the synthesis of amino-terminated PLGA copolymers.

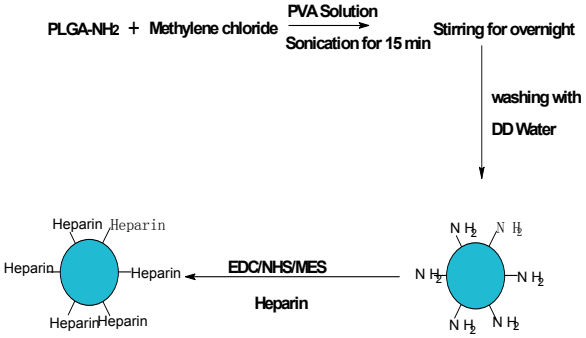

Fig.2. A schematic diagram for the preparation of PLGA microspheres and the conjugation of heparin to the surface of the PLGA microspheres.

Table 1. Table Type Styles

Group	Information of the delivery system			
	Table column subhead	*Heparin free form [μg]*	*HCPN [mg]*	*bFGF [μg]*
Group I	5	5	-	1
Group II	-	-	3	1
Group III	5	-	3	1

2.3. Determination of bFGF release from M-collagen in vitro

The efficiency of bFGF complexation to HCPMs was

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determined with enzyme-linked immunosorption assay (ELISA). HCPMs and bFGF were mixed in saline solution. After 1 h, HCPMs were collected by centrifugation at 10000 rpm for 10 min. The amounts of bFGF were determined with an ELISA kit. The kinetics of bFGF release from HCPMs that suspended in collagen, HCPMs alone, and M-collagen containing heparin in free form were determined (Table 1). Each of delivery systems was immerged in 2 ml microcentrifuge tubes containing 1.5 ml buffered saline and sodium azide. The samples were incubated at 37 °C with continuous agitation. At various time points, the supernatant was withdrawn and buffer was replenished. The amounts of bFGF in the supernatants were determined with an ELISA kit. After the appropriately diluted samples were added 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 and substrate were added and incubated for 20 min. The enzyme reaction was stopped by adding an acidic solution. The absorbance of the samples was read at 450 nm using an ELISA plate reader. The amount of bFGF was determined from a calibration curve based on known concentrations of bFGF.

2.4. bFGF bioactivity assay

The bioactivity of bFGF released from HCPMs suspended in M-collagen and from M-collagen gels containing heparin in free form was assessed in vitro by determining its ability to stimulate the proliferation of HUVECs cultured in Cell Counting Kit-8 (CCK-8). BFGF was added to the HCPMs suspended in M-collagen and the M-collagen containing heparin in free form. Cells were seeded in each well tissue culture plates and the bFGF delivery systems were fixed on a culture insert. On days 1, 3, 5, 7, and 9, cell numbers were measured using a hemocytometer. As a positive control, bFGF was added daily to a HUVEC culture in CCK-8 at M-collagen. HUVECs cultured in CCK-8 without bFGF served as a negative control. The medium was changed every 3 days.

3. Results

3.1. Preparation of heparin-conjugated PLGA microspheres

Ninhydrin colorimetric method showed bluish-purple after drop into Sample. Furthermore, as negative control group, MPLGA showed bluish-purple never. As positive control group, ethylenediamine showed bluish-purple too. THF did showed bluish-purple as blank control group. we conclude that ethylenediamine react with MPLGA to form PLGA-NH_2.

The amounts of microspheres were made from the PLGA–NH_2 conjugated heparin. A scanning electron microphotograph of the fabricated HCPMs showed that the microspheres were spherical, discrete particles without aggregation (Fig. 3A). A scanning electron microphotograph of the fabricated HCPMs suspended in M-Collagen showed that microspheres immobilized on M-Collagen closely (Fig. 3B).

3.2. Content of grafted heparin

Using these conditions, pre-activation of heparin with EDC and NHS for 5 to 30 min resulted in maximal heparin immobilization (Fig. 4A). Using a pre-activation time of 10 min and a molar ratio EDC : NHS : heparin-carboxylic acid groups (Hep-COOH) of 0.4 : 0.24 : 1.0, the amount of immobilized heparin increased when the molar ratio of heparin to free primary amino groups of collagen (Coll-NH_2) was increased, leveling oil above a ratio of 2 (Fig. 4B). Heparin immobilization using a molar ratio of heparin: Coll-NH2 of 2.0 (10 min pre-activation, EDC: Hep-COOH=0.4) was maximal at a molar ratio of NHS to EDC in between 0.4 to 0.6 (Fig. 4C). At a fixed molar ratio of NHS : EDC of 0.6 (10 min pre-activation, EDC : Hep-COOH=0.4), the amount of immobilized heparin increased with increasing molar ratio of EDC to Hep-COOH , to a maximum of approximately 5.5% heparin (w/w) at a ratio of 2 (molar ratio EDC : NHS : Hep-COOH=2.0 : 1.2 : 1.0) (Fig. 4D). Based on these results a standard procedure for heparin immobilization to M-Collagen was adopted, using 2% (w/v) heparin solution (pH 5.60), a fixed molar ratio of NHS to EDC of 0.6, a variable molar ratio of EDC: Hep-COOH of 0 to 2.0, heparin activation for 10 min, a molar ratio of heparin to Coll-NH_2 of 2.0, and 2 h of immobilization reaction. In further experiments, the amount of heparin immobilized to M-Collagen was predetermined by the molar ratio of EDC to Hep-COOH used for immobilization.

3.3. Release kinetics of bFGF

The curve of bFGF release from HCPMs suspended in M-collagen was determined by ELISA and compared with those from HCPMs and M-collagen (Fig. 5). The release of bFGF from M-collagen was more rapid than that from HCPMs alone or from HCPMs suspended in M-collagen. The bFGF release from the M-collagen was sustained for only 8 days. In contrast, the bFGF release

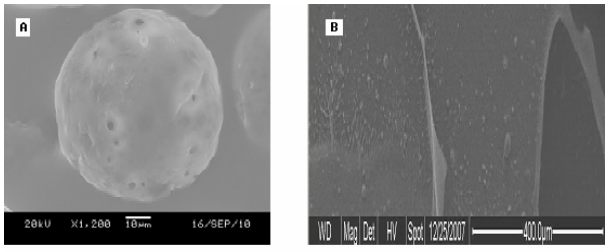

Fig.3. Scanning electron micrograph of HCPMs and HCPMs suspended in Modified Collagen

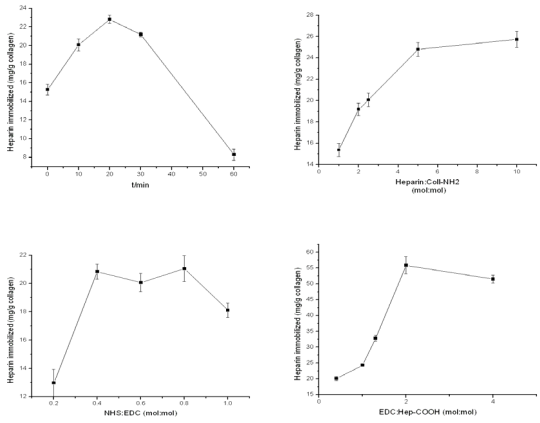

Fig.4. Immobilization of heparin to collagen as a function of pre-activation time of heparin(A), molar ratio of heparin to free primary amino groups in collagen(B), molar ratio of NHS to EDC (C), and molar ratio of EDC to heparin carboxylic acid groups (D).

from HCPMs was sustained for 21 days. Importantly, the bFGF release was further prolonged by employing HCPMs suspended in M-collagen, it could sustained for 30 days.

3.4. Bioactivity of bFGF released from delivery systems

To determine whether the bFGF released from HCPMs in collagen is bioactive, the biological activity of the bFGF was evaluated by measuring its ability to stimulate the growth of HUVECs cultured in a medium containing bFGF-loaded HCPMs suspended in M-collagen (Fig. 6). The HUVECs showed no cell growth and decreased in cell number in the basal medium without bFGF. The daily addition of bFGF in a free form to the basal medium stimulated the HUVECs growth. The cell growth for 15 days in the medium containing bFGF-loaded HCPMs suspended in M-collagen was not different from that in the medium to which bFGF in a free form was added daily. This indicates that the bFGF released from HCPMs in M-collagen for up to 10 days was bioactive. bFGF loaded collagen exhibited cell growth that was not significantly different from that of the bFGF-loaded HCPMs and M-collagen for the first 7 days, but exhibited a decrease in cell number.

4. Discussion

bFGF has angiogenic and mitogenic properties, and has ability to regenerate tissues including bone [13,14], blood vessels [15-18], and skin [19,20]. To increase the treatment effectiveness of bFGF, it would be necessary to develop a durative delivery system for bFGF [21]. This study shows that bFGF release can be sustained using HCPMs suspended in M-collagen for more than 4 weeks. Since most medicine have a short half-life in vivo and a risk of carcinogenic activity after systemic delivery. The delivery system of HCPMs in collagen developed in this study can deliver bFGF over 4 weeks. In this study, the bFGF delivery system using HCPMs in M-collagen showed an enhanced generation of HUVECs in vitro.

The delivery system of HCPMs suspended in M-collagen exhibited zero-order, long-term release of bFGF. BFGF is released from M-collagen by a simple diffusion-controlled mechanism, which exhibits a short-term release of bFGF with an initial burst (Fig. 5). The bFGF directly incorporated into HCPMs in M-collagen would be released by a combination of the mechanisms of dissociation from HCPMs and diffusion through M-collagen. The zero-order, long-term deliver of bFGF from HCPMs suspended in M-collagen may be attributed to the combination of the two release mechanisms. BFGF is released from heparin suspended in M-collagen [22,23]. The bFGF release rate may be increased in vivo because M-collagen degraded rapidly in vivo and the release of bFGF from HCPMs alone was more rapid than that from HCPMs in M-collagen.

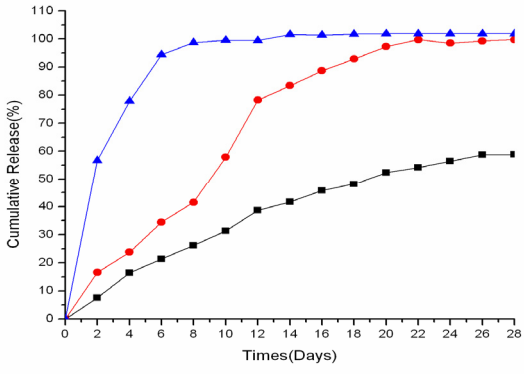

Fig.5. Profiles of bFGF release from M-Collagen containing heparin in free from (▲), HCPMs suspended in M-Collagen (■), and HCPMs (●).

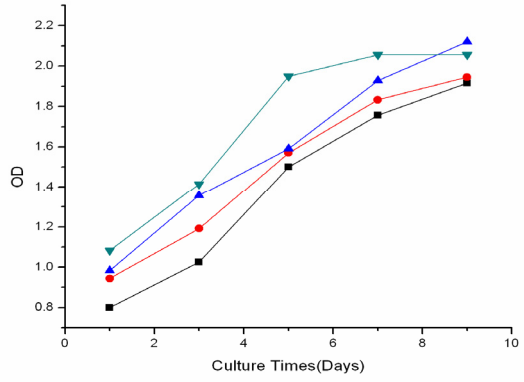

Fig.6. The growth kinetics of HUVEC cultured in (■) CCK-8, (●) CCK-8 with daily additions of bFGF and

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heparin in free form, (▲) CCK-8 containing bFGF-loaded M-Collagen, or (▼) CCK-8 containing bFGF-loaded HCPMs suspended in M-Collagen.

5. Conclusions

This study demonstrates that a delivery system of HCPMs in M-collagen can sustain delivery bFGF for more than 4 weeks at almost zero-order. The bFGF released by the delivery system holded its biological activity. The controllable delivery system for bFGF developed in this study may provide a strong modality for a variety of therapeutic measure, such as wound healing, and bone regeneration. This delivery system could also be applied to release dual or multiple angiogenic factors that have affinities for heparin, such as cell hepatocyte growth factor, vascular endothelial growth factor, epidermal growth factor, and bone morphogenetic protein [24], which could importantly enhance angiogenesis [25].

6. Acknowledgements

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References

[1] Hang Lin,Yannan Zhao,Wenjie Sun,et al. The effect of crosslinking heparin to demineralized bone matrix on mechanical strength and specific binding to human bone morphogenetic protein-2. Biomaterials Vol. 1189-1197(2008), pp. 29.
[2] Chang Yaoa,Marta Markowicz,Norbert Pallu,et al.The effect of cross-linking of collagen matrices on their angiogenic capability.Biomaterials Vol. 66–74 (2008), pp. 29.
[3] Yu Hanglong, Peng Hong, Sun Huayan, et al. Military Surgeon Advanced Study Institute Journal Vol. 73-75 (2009), pp. 30.
[4] Tabata Y, Hijikata S, Ikada Y. Enhanced vascularization and tissue granulation by basic fibroblast growth factor impregnated in gelatin hydrogels. J Control Release Vol. 189-99 (1994), pp. 31.
[5] Liu LS, Ng CK, Thompson AY, Poser JW, Spiro RC. Hyaluronate-heparin conjugate gels for the delivery of basic fibroblast growth factor (FGF-2). J Biomed Mater Res Vol. 128-35 (2002), pp. 62.
[6] Oju Jeon, Sun-Woong Kang, et al. Biomaterials Vol. 1598-1607 (2002), pp. 27.
[7] Cao Xuebo, Wang Yuanliang, et al. Polymer Materials Science And Engineering Vol. 18 (2002), pp. 1.
[8] Luo Yanfeng, Wangyuanliang, et al. Polymer Materials Science And Engineering Vol. 21 (2005), pp. 2.
[9] Mikos A G, Thorsen A J, et al. Polymer Vol. 1068 (1994), pp. 35.
[10] Gilles MA, Hudson AQ, Borders CL. Stability of water-soluble carbodiimides in aqueous solutions. Anal Biochem Vol. 184-244 (1990), pp. 29.
[11] Besselink GAJ, Beugeling T, Bantjes A. N-Hydroxysuccinimideactivated glycine-sepharose. Appl Biochem Biotech Vol. 227-246 (1993), pp. 43.
[12] Ramachandran GN. Chemistry of collagen. London: Academic Press, 1967.
[13] Sha Huang,Yijuan Wang, Tang Liang,et al.Fabrication and characterization of a novel microparticle with gyrus-patterned surface and growth factor delivery for cartilage tissue engineering. Materials Science and Engineering Vol. 249 (2008), pp. 6.
[14] Hang Lin,Yannan Zhao,Wenjie Sun,et al. The effect of crosslinking heparin to demineralized bone matrix on mechanical strength and specific binding to human bone morphogenetic protein-2. Biomaterials Vol. 1189-1197 (2008), pp. 29.
[15] Hao Yuewen,Liu Ying,Sun Lijun, et al. Clinical Radiology Journals Vol. 278-281 (2008), pp.27.
[16] Wang Ying, Zhao Jian, Kong Xiangrong, et al. Cadiovascular recovery medicine Journals Vol. 142-154 (2008), pp. 17.
[17] Wei Jiang,Hailun Wang,Faguang Jin,et al.Improved wound healing in pressure-induced decubitus ulcer with controlled release of basic fibroblast growth factor. Journal of Alloys and Compounds Vol. 508–514 (2008), pp. 459.
[18] Chang Yaoa,Marta Markowicz,Norbert Pallu,et al.The effect of cross-linking of collagen matrices on their angiogenic capability.Biomaterials Vol. 66–74 (2008), pp. 29.
[19] Sha Huang,Tianzheng Deng,Yijuan Wang,et al.Multifunctional implantable particles for skin tissue regenerationregeneration: Preparation, characterization, in vitro and in vivo studies. Acta Biomaterialia Vol. 1057–1066 (2008), pp. 4.
[20] Zexu Gu,Liang Kong,Xue Feng,et al.Synthesis and characterization of PLGA–gelatin complex with growth factor incorporation as potential matrix. Journal of Alloys and Compounds Vol. 150 (2008), pp. 06.
[21] Sakiyama-Elbert SE, Hubbell JA. Development of fibrin derivatives for controlled release of heparin-binding growth factors. J Control Release Vol. 389–402 (2000), pp. 65.
[22] Jeon O, Ryu SH, Chung JH, Kim BS. Control of basic fibroblast growth factor release from fibrinwith heparin and concentrations of fibrinogen and thrombin. J Control Release Vol. 249-259 (2005), pp. 10.
[23] Saksela O, Rifkin DB. Release of basic fibroblast growth factorheparan sulfate compleses from endothelial cells by plasminogen activator-mediated proteolytic activity. J Cell Biol Vol. 767-775 (1990), pp. 110.
[24] Ashikari-Hada S, Habuchi H, Kariya Y, Itoh N, Reddi AH, Kimata K. Characterization of growth factor-binding structures in heparin/heparan sulfate using an octasaccharide library. J Biol Chem Vol. 46-54 (2004), pp. 279.
[25] Richardson TP, Peters MC, Ennett AB, Mooney DJ. Polymeric system for dual growth factor delivery. Nat Biotechnol Vol. 102-124 (2001), pp. 19.