International Journal of Natural and Engineering Sciences 5 (3): 65-70, 2011
ISSN: 1307-1149, E-ISSN: 2146-0086, www.nobel.gen.tr

Basic Fibroblast Growth Factor Retains Its Biological Activity *In Vitro* When Delivered Through Degradation of Gelatin Microspheres

Aylin ŞENDEMİR ÜRKMEZ[1]* Russell D. JAMISON[2]

[1] Department of Bioengineering, Faculty of Engineering, Ege University, Bornova, 35100, Izmir, TURKEY

[2] Departments of Biomedical Engineering and Chemical and Life Sciences Engineering, Virginia Commonwealth University, Richmond, Virginia 23284-3068, USA

***Corresponding Author**

e-mail: aylin.sendemir@ege.edu.tr

Received: August 23, 2011
Accepted: September 03, 2011

Abstract

In designing delivery systems for growth factors and other protein drugs, it is important to know what percentage of the loaded protein will be delivered in biologically active form. This work evaluates the release system of basic fibroblast growth factor (bFGF) through gelatin microspheres. The results indicate that there is no detectable loss of mitogenic activity of the growth factor on fibroblastic cells after delivery.

Keywords: Gelatin microspheres, biological activity, controlled release

INTRODUCTION

Basic fibroblast growth factor (bFGF) is a potent angiogenic factor that promotes endothelial cell migration, proliferation, and capillary differentiation [1-3]. It regulates proliferation and differentiation of several other cell types including mesenchymal cells, chondrocytes, osteoblasts, fibroblasts, neuronal and smooth muscle cells [1-4]. It contributes to wound healing by speeding granulation tissue formation and collagen accumulation [5, 6] and enhances bone and cartilage formation in early phases of healing in a dose dependent manner [2, 7-11]. Mechanically damaged endothelial cells, burned tissues, and macrophages activated during the inflammatory response secrete bFGF as well [5]. FGFs are characterized by their affinity for the glycosaminoglycans (GAGs). It is believed that bFGF is stored in vivo in the extracellular matrix as an FGF-GAG complex. In the stored form, it is biologically inert and the cells surrounding it are quiescent. This complex protects the growth factor in vivo, and is released from the matrix during injury and subsequent healing by heparinase and proteases like plasmin [12-16]. bFGF is a relatively unstable protein and loses its activity if put in contact with certain solvents, exposed to alkaline conditions, or stored at room temperature [16-18].

Although bolus delivery of growth factors gives good results in small animals, it shows limited efficacy in clinical trials [19-23]. The main problem with bolus delivery is the rapid denaturation of bFGF in physiological conditions. The half-life of bFGF was reported as 6 minutes when administered intravenously [24]. Therefore in clinical applications, bFGF is usually delivered bound to heparin to prevent denaturation and inactivation [25, 26]. The biological activity of bFGF is partially or totally lost when synthetic delivery vehicles like poly(lactide-co-glycolide) and ethylene-vinyl acetate (EVAc) are used due to denaturation of the protein during polymer processing conditions including heat and organic solvents [26-30]. Tabata et.al. have successfully demonstrated the sustained release of biologically active bFGF using gelatin sheets [31-33] and microspheres [34]. Gelatin is denatured collagen. It can be processed to take acidic and basic forms. Acidic gelatin with an isoelectric point of 5.0 is obtained through an alkaline process by hydrolysis of amide groups of collagen [35]. Through ionic complexation, acidic gelatin can mimick the function of extracellular matrix GAGs that bFGF is complexed with in physiological conditions. It thereby retains the biological activity of the growth factor longer than bolus injection [36, 37]. Both in vivo and in vitro experiments show that bFGF only complexes with the acidic form of gelatin. Sorption decreases with increased solution ionic strength. Both properties suggest that the main interaction between the two is electrostatic [31, 32, 37]. The release of the growth factor after an initial burst of unbound protein is substantially controlled by the enzymatic degradation of gelatin in vivo [31, 32]. The degradation of gelatin involves a complex mechanism yet to be elucidated, most likely involving a combination of different enzymes and specific physiological conditions. Recent studies with different collagenases have shown that in vitro collagenase type IV gives the closest profile to the in vivo degradation profile of gelatin (unpublished data).

Although *in vivo* experiments have shown clearly that the delivery of bFGF by gelatin prevents complete loss of its biological activity, no study has measured the extent to which it is preserved. A direct evaluation of the amount of biological activity retained will provide valuable insight in designing the delivery systems and determining dosage. This study aims to answer this question by directly comparing the mitogenic effect of bFGF on fibroblastic cells *in vitro* with and without a delivery system of gelatin microspheres. In order to prevent any loss of activity in time, an accelerated degradation process has been designed and utilized for this purpose. Free and bound bFGF were introduced to fibroblastic cells at the same time and the release of bound bFGF was achieved through enzymatic degradation of gelatin microspheres. No activity loss of bFGF on fibroblastic cell proliferation was detected.

MATERIALS AND METHODS

Cell Culture

Swiss 3T3 mouse embryonic fibroblasts were purchased from American Type Culture Collection (ATCC, Manassas, VA). They were cultured according to supplier's recommendations in DMEM growth media, containing 10% newborn calf serum (NCS) and 1% antibiotic/antimycotic mixture at 37°C in a 5% CO_2 humidified environment. After 80% confluence, cells were harvested by gentle digestion with 0.05% trypsin/EDTA, counted with a hemacytometer, suspended in fresh media and then seeded onto standard flat bottom 96-well microtiter plates at a density of $5x10^3$ cells/well. Assay for bioactivity was modified from [28]. Briefly, after one day of incubation in growth media, each well was washed with phosphate buffered saline (PBS) and supplied with 200 mL of serum free media. The cells were serum deprived for an additional 24 hours before the media were replaced with the test solutions.

Preparation of Gelatin Microspheres

Gelatin with an isoelectric point (IEP) of 5.0 was a gift from Professor Tabata (Kyoto University, Kyoto, Japan). Gelatin microspheres were fabricated by a precision particle fabrication (PPF) technique described elsewhere [38, 39]. Briefly, a 10% aqueous gelatin solution was passed through a nozzle with a carrier stream. As the nozzle was excited by acoustic vibration, the stream of gelatin was broken into uniform droplets. The droplets were collected in an oil bath. Resulting gelatin microspheres were then washed and homogenized three times in cold acetone and allowed to dry overnight at 4ºC. Twenty milligrams of dried microspheres were crosslinked with 500ml 25% glutaraldehyde for 8 hours at 4ºC. The reaction was then terminated with 100mM glycine for 1hr at 37ºC. After three one hour washes in de-ionized water at 37ºC, the microspheres were collected by centrifugation, frozen in liquid nitrogen for 30 minutes and placed in the lyophilizer for 72hr.

Degradation of Gelatin Microspheres

In order to determine the proper concentration of collagenase type IV (gelatinase) (Sigma Chemicals, St. Louis, MO) to degrade microspheres in two days, different concentrations were tested. Since the calcium concentration plays a crucial role in gelatin degradation, $CaCl_2$ was added to Dulbecco's PBS (DPBS) in order to achieve an equal concentration of $CaCl_2$ (200mg/L) with the DMEM cell culture medium. A gelatinase stock solution of 100U/mL was prepared in this modified DPBS (mDPBS). Two milligrams of microspheres were placed in polystyrene centrifuge tubes, and 5mL solutions of 0 – 10% gelatinase in mDPBS were added onto each tube and pipetted gently for mixing. All groups were tested in five replicates. The tubes were incubated at 37ºC, and 300mL of the supernatant was taken after 2, 4, 8, 12, 24 and 48 hours. The gelatin amount in the supernatant was determined in two replicates from each tube using Micro BCA™ Protein Assay Kit (Pierce Biotechnology, Rockford, IL). The absorbance was measured at 562nm by a microplate reader (Synergy™ HT), and the gelatin concentrations were calculated using a curve prepared by BSA standards.

Loading of Gelatin Microspheres with bFGF

The recombinant human bFGF (R&D Systems, Minneapolis, MN) was hydrated to the desired concentrations with mDPBS. Two micrograms of the lyophilized microspheres was placed in low protein binding microcentrifuge tubes (Eppendorf, Hamburg, Germany). Twenty microliters of desired bFGF solution or mDPBS was dropped onto the microspheres and allowed to absorb for 2 hours at room temperature. This amount was much smaller than the required solution for fully hydrating the microspheres. After absorption, the microspheres were suspended in required media solutions with repeated pipetting before application onto cells.

Administration of Experimental Groups to Cells

Three different sets of experiments were performed in order to 1) confirm the proliferative effect of different concentrations of bFGF on cells; 2) verify the effects of different concentrations of gelatinase on cells; and 3) determine the amount of biological activity retained after release of bFGF through gelatin degradation. The experimental groups are summarized in Tables 1-3. In each experiment, the serum deprived cells on 96-well plates were washed with PBS and the media were replaced by 200mL of the experimental media solution. The cells were incubated at 37°C in a 5% CO_2 humidified environment for two days before testing for proliferation.

Table 1. Test groups for Experiment 1. This experiment is performed to determine the proliferative effect of different concentrations of bFGF on cells. All solutions are in DMEM.

Group:	Free bFGF (ng/mL)	
1	-	(-) control
2	1	
3	5	
4	10	

Table 2. Test groups for Experiment 2. This experiment is performed to determine the effects of gelatinase on cells. All solutions are in DMEM.

Group:	Gelatinase (%)	
1	-	(-) control
2	2.5	
3	5	
4	7.5	
5	10	
6	25	

Table 3. Test groups for Experiment 3. This experiment is performed to determine the amount of biological activity retained after bFGF release through gelatin degradation. All solutions are in DMEM.

Group	bFGF (5ng/mL)	Gelatin Microspheres (100mg)	Gelatinase (5%)	NCS	
1	-	-	-	-	(-) control
2	-	-	-	10%	(+) control
3	Free	-	-	-	
4	Free	-	+	-	
5	Free	+	+	-	
6	Gelatin bound	-	-	-	
7	Gelatin bound	-	+	-	
8	-	-	+	-	
9	-	+	-	-	
10	-	+	+	-	

Determination of Cell Proliferation

Viable cell number was measured with the colorimetric (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) (MTT) assay. At the end of the experimental period, cells on 96-well plates were gently rinsed with PBS. MTT was mixed with PBS at a concentration of 5mg/mL to make the MTT reagent. MTT reagent was mixed with growth media at a ratio of 1:10, and 100mL was added to each cell culture well and incubated for 4 hours at 37°C. After incubation, 100mL of 0.04 N HCl in isopropanol was added to each well. The solution was mixed by vigorous pipetting to dissolve the formazan crystals. Absorbance was measured by a microplate reader (SynergyTM HT, BioTek Instruments, Inc. Winooski, VT) at 570nm and a reference wavelength of 630nm. Absorbance was converted to cell number utilizing a calibration curve produced with known cell numbers.

Statistical Analysis

Differences between groups were analyzed by one-way analysis of variance (ANOVA) using Fisher's Least Significant Difference tests for multiple comparisons with a critical significance value of 0.05. All analyses were performed using the Minitab statistical software program (Minitab Inc., State College, PA).

RESULTS

Degradation of Gelatin Microspheres

The gelatin microspheres were totally degraded by gelatinase in two days with the concentrations of 2.5% and above (Figure 1). Since the experiments with the cells would be performed under static conditions, the degradation tests were also performed under static conditions, but with a 20 fold higher quantity of microspheres. This amount was chosen in order to increase the precision and ease of measurement as well as to remain within the detection limits of the protein assay.

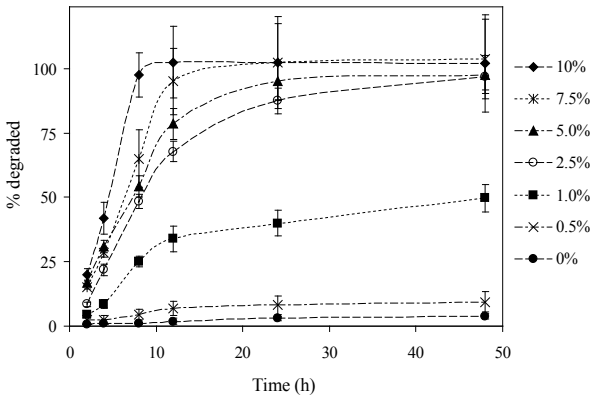

Figure 1. Degradation of gelatin microspheres with different concentrations of gelatinase. At concentrations above 2.5% (v/v), the degradation of the gelatin microspheres was completed within two days. Data are shown as means ± SD (n=5 for each point).

Effects of bFGF on Cell Proliferation

All three concentrations of bFGF tested showed an increase on cell proliferation compared to serum-free DMEM control (Figure 2), confirming the stimulatory effect of bFGF on Swiss 3T3 cell proliferation. There was no statistically significant difference among the concentrations used, suggesting the existence of a threshold concentration for mitogenic stimulation.

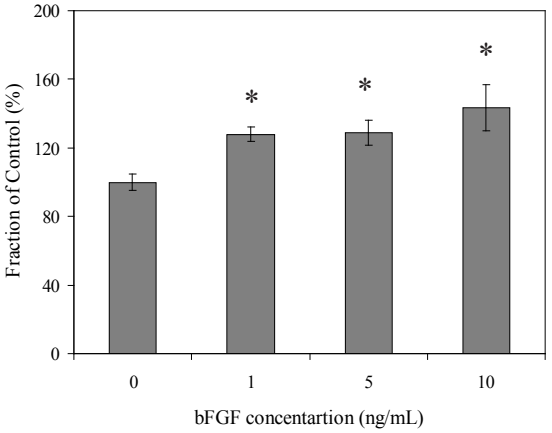

Figure 2. All three concentrations of bFGF tested showed significant increase in cell proliferation in serum free media compared to control. There was no significant difference among the concentrations used. Data show the cell numbers in each group as the percent fraction of the control group ± SD (n=5). *Significant difference compared to control, $p<0.05$.

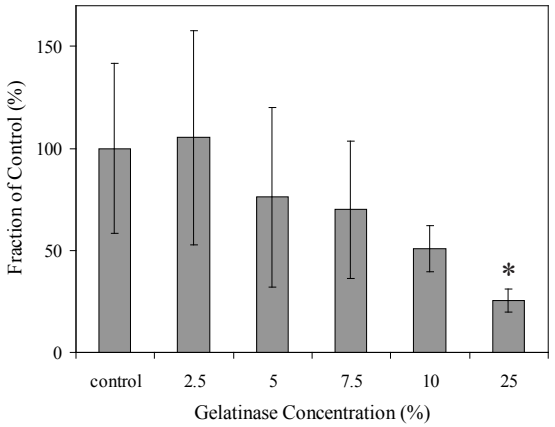

Figure 3. Among the concentrations of gelatinase tested, only 25% showed significant difference in cell number with the control group in serum free media. Data show the cell numbers in each group as the percent fraction of the control group ± SD (n=5). *Significant difference compared to control, $p<0.05$.

Effects of Gelatinase on Cell Viability

The addition of gelatinase into serum-free media did not induce a detrimental effect on cell viability for gelatinase concentration of 10% and below. For the concentrations tested, only 25% gelatinase showed a significant reduction in cell number compared to controls (Figure 3).

Retained Biological Activity of bFGF after Release

In light of the previous experiments, bFGF concentration of 5ng/mL and gelatinase concentration of 5% were chosen for the biological activity test. These values were within the limits for bFGF to induce cell proliferation, for the gelatin microspheres to degrade before two days, and for the gelatinase not to influence the cell viability. It can be seen in Figure 3.4 that there was no significant difference between the groups where the proliferation was induced by bFGF, regardless of whether the bFGF was administered in free form or through degradation of gelatin microspheres. These groups showed a significant increase in cell number compared to (-) control and all other groups containing gelatin microspheres and/or gelatinase in

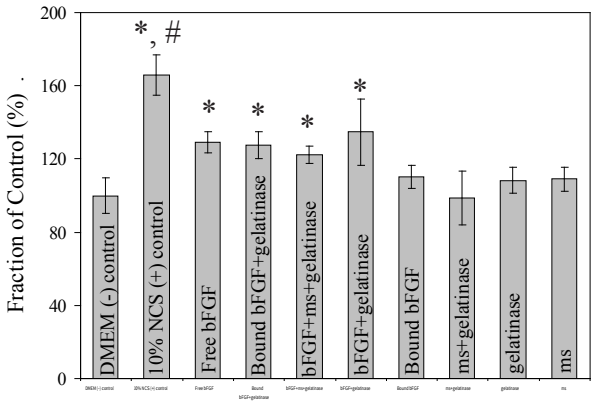

Figure 4. All experimental groups that received bFGF showed significantly higher proliferation compared to those that did not receive bFGF, except (+) control group. There was no significant difference in cell proliferation between the groups that received bFGF with or without the gelatin delivery system. The gelatin bound bFGF was not released without degradation by gelatinase, therefore that group did not receive any bFGF. There was no significant difference among the groups that received only microspheres and/or gelatinase compared to (-) control group. Data show the cell numbers in each group as the percent fraction of the (-) control group ± SD (n=5). *Significant difference compared to (-) control, #significant difference compared to bFGF receiving groups, $p<0.05$.

serum-free media. When there was no gelatinase present for microsphere degradation, bound bFGF did not produce any effect on cell proliferation. Administration of bFGF did not induce cell proliferation as much as 10% NCS that was used as the (+) control. Accelerated degradation of gelatin microspheres using gelatinase did not induce any detrimental effects on the cells, or bFGF. The gelatin microspheres with or without gelatinase did not have any effect on the cell proliferation (Figure 4).

DISCUSSION

In designing delivery systems for growth factors and other protein drugs, it is important to know what percentage of the loaded protein will be delivered in biologically active form. In order to achieve sustained, efficient delivery, the delivery system must act like ECM to stabilize and protect the loaded protein from denaturation, prevent its interactions with the extracellular environment before required release time, and provide the desired release rate through engineered material properties. This study demonstrates the ability of gelatin microspheres to preserve virtually all biological activity of bFGF *in vitro* when released through enzymatic degradation of gelatin.

FGFs were initially characterized as a mitogen for 3T3 fibroblastic cells [1]. Later it was discovered that it is mitogenic for all mesoderm derived cells [3], although fibroblastic cells are still most frequently used to test the biological activity of bFGF [13, 16, 26, 28, 40, 41]. The main effect of bFGF on cells is to act as a "competence factor" that forces cells to leave their resting phase and to start DNA production leading to proliferation [2, 18]. Our results confirm this observation, i.e., when introduced in a free form to serum deprived fibroblastic cells, bFGF induces proliferation. When bFGF is loaded onto gelatin microspheres as described, electrostatic interaction between the two protects bFGF from denaturation, simulating the sequestration role of ECM. bFGF released from gelatin microspheres showed an inductive effect on cell proliferation identical to that of free bFGF. A previous study had shown no effect on proliferation of baby hamster kidney (BHK) cells when bFGF is loaded onto IEP5 gelatin sheets at similar concentrations and loading method [42]. In that study, the gelatin sheets were not degraded, and the release of unbound bFGF was tested. Our results are parallel with their observation, indicating that when there is no gelatinase in wells, there is no difference in cell proliferation behavior. It is possible that some unbound bFGF is released immediately without any degradation but this amount must be lower than the threshold value that would stimulate the cells.

During the alkaline process, amide groups of collagen are hydrolyzed making the resulting gelatin negatively charged with a large number of carboxyl groups [35]. The bFGF molecule is small enough (MW 17,000) to freely diffuse into gelatin, and has been shown to impregnate into and ionically interact with IEP5 gelatin hydrogels when loaded as described [31, 37]. When folded in its natural confirmation, bFGF has an unusual cluster of positively charged residues on one side of the protein, which also gives its latent instability [16]. It is likely that the negative charge density on IEP5 gelatin stabilizes the positively charged residues on bFGF possibly also altering the molecular confirmation. When bFGF is delivered as bound to heparin, the radius of diffusion at the extracellular environment increases due to reduced rate of binding to the bFGF receptors on cells [14]. We have no evidence on whether the heparin binding domains and the gelatin complexation domain(s) of bFGF are the same, but our results suggest that complexation with gelatin might similarly reduce the interactions of bFGF with cells until degradation. This property would increase the controllability of the protein delivery through engineering the degradation rate of gelatin.

It is important to note that our *in vitro* degradation system is not intended to model the *in vivo* degradation of gelatin where several other enzymes are contributing to the process. On the contrary, we used an accelerated degradation system in order to use a well established cellular assay to test biological activity. Our results indicate that the biological activity of bFGF is retained when delivered by gelatin microspheres to fibroblastic cells *in vitro*. Although the inherent differences between the *in vivo* and *in vitro* systems prevent us to extend all the findings to *in vivo* conditions, this study demonstrates the efficacy of gelatin microspheres as bFGF carriers for potential drug delivery systems and tissue engineering applications.

CONCLUSIONS

Gelatin microspheres are potentially attractive delivery vehicles for bFGF, since the protein delivery rate can be controlled by controlling the microsphere degradation rate. Our present results demonstrate that when delivered through degradation of gelatin, bFGF show no detectible loss of activity on Swiss 3T3 fibroblastic cell proliferation *in vitro*. Although it is not possible to extrapolate this result to *in vivo* conditions due to inherent differences between the two systems, it presents strong evidence of the preservation of biological activity with gelatin delivery systems carrying bFGF.

Acknowledgments

Authors would like to thank Prof. Yasuhiko Tabata from Kyoto University for sharing his expertise on gelatin delivery systems.

REFERENCES

[1] Engleka KA, Maciag T. 1999. Molecular Mechanisms of the Fibroblast Growth Factor Family. In: Simons, M, Simons, M. Angiogenesis and Cardiovascular Disease. New York: Oxford University Press: 79-100.

[2] Millis DL. 1999. Bone and None-Bone-Derived Growth Factors and Effects on Bone Healing. Veterinary Clinics of North America: Small Animal Practice; 29: 1221-1246.

[3] Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G. 1987. Structural Characterization and Biological Functions of Fibroblast Growth-Factor. Endocrine Reviews; 8: 95-114.

[4] Kempen DHR, Creemers LB, Alblas J. 2010. Growth Factor Interactions in Bone Regeneration. Tissue Engineering Part B-Reviews; 16(6): 551-566.

[5] Xie JL, Bian HN, Qi SH. 2008. Basic fibroblast growth factor (bFGF) alleviates the scar of the rabbit ear model in wound healing. Wound Repair And Regeneration; 16(4): 576-581 .

[6] Lee KY, Peters MC, Mooney DJ. 2003. Comparison of Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor on Angiogenesis in Scid Mice. Journal of Controlled Release; 87: 49-56.

[7] Deng T, Huang S, Zhou S. 2007. Cartilage regenera-tion using a novel gelatin-chondroitin-hyaluronan hybrid scaffold containing bFGF-impregnated microspheres. Journal of Microencapsulation: 24(2): 163-174.

[8] Draenert GF, Draenert K, Tischer T. 2009. Dose-dependent osteoinductive effects of bFGF in rabbits Growth Factors; 27(6): 419-424.

[9] Qu D, Li J, Li Y. 2011. Angiogenesis and osteogenesis enhanced by bFGF ex vivo gene therapy for bone tissue engineering in reconstruction of calvarial defects. Journal of Biomedical Materials Research; 96A(3): 543-551.

[10] Jiang X, Zou S, Ye B. 2010. bFGF-Modified BMMSCs enhance bone regeneration following distraction osteogenesis in rabbits. Bone; 46(4): 1156-1161.

[11] Akman AC, Tigli RS, Gumusderelioglu M. 2010. bFGF-loaded HA-chitosan: A promising scaffold for periodontal tissue engineering. Journal of Biomedical Materials Research; 92A(3): 953-962.

[12] Baird A. 1994. Potential Mechanisms Regulating the Extracellular Activities of Basic Fibroblast Growth Factor (Fgf-2). Molecular Reproduction and Development; 39: 43-48.

[13] Rifkin DB, Moscatelli D. 1989. Recent Developments in the Cell Biology of Basic Fibroblast Growth-Factor. Journal of Cell Biology; 109: 1-6.

[14] Flaumenhaft R, Moscatelli D, Rifkin DB. 1990. Heparin and Heparan-Sulfate Increase the Radius of Diffusion and Action of Basic Fibroblast Growth-Factor. Journal of Cell Biology; 111: 1651-1659.

[15] Taipale J, KeskiOja J. 1997. Growth Factors in the Extracellular Matrix. Faseb J; 11: 51-59.

[16] Wang YJ, Shahrokh Z, Vemuri S, Eberlein G, Beylin I, Busch M. 1996. Characterization, Stability and Formulations of Basic Fibroblast Growth Factor. In: Pearlman, R, Wang, YJ, Pearlman, R, Wang, YJ. Formulation, Characterization, and Stability of Protein Drugs. New York: Plenum Press: 141-180.

[17] Estape D, van den Heuvel J, Rinas U. 1998. Susceptibility Towards Intramolecular Disulphide-Bond Formation Affects Conformational Stability and Folding of Human Basic Fibroblast Growth Factor. Journal of Biochemistry; 335: 343-349.

[18] Burgess WH, Maciag T. 1989. The Heparin - Binding (Fibroblast) Growth Factor Family of Proteins.; 58: 575-606.

[19] Ennett AB, Mooney DJ. 2002. Tissue Engineering Strategies for *in vivo* Neovascularisation. Expert Opin Biol Th; 2: 805-818.

[20] Post MJ, Laham R, Sellke FW, Simons M. 2001. Therapeutic Angiogenesis in Cardiology Using Protein Formulations. Cardiovascular Research; 49: 522-531.

[21] Epstein SE, Fuchs S, Zhou YF, Baffour R, Kornowski R. 2001. Therapeutic Interventions for Enhancing Collateral Development by Administration of Growth Factors: Basic Principles, Early Results and Potential Hazards. Cardiovascular Research; 49: 532-542.

[22] Ferrara N, Alitalo K. 1999. Clinical Applications of Angiogenic Growth Factors and Their Inhibitors. Nature Medicine; 5: 1359-1364.

[23] Zisch AH, Lutolf MP, Hubbell JA. 2003. Biopolymeric Delivery Matrices for Angiogenic Growth Factors. Cardiovascular Pathology; 12: 295-310.

[24] Edelman ER, Nugent MA, Karnovsky MJ. 1993. Perivascular and Intravenous Administration of Basic Fibroblast Growth-Factor - Vascular and Solid Organ Deposition. Proceedings of the National Academy of Sciences of the United States of America; 90: 1513-1517.

[25] Nomi M, Atala A, Coppi PD, Soker S. 2002. Principles of Neovascularization for Tissue Engineering. Molecular Aspects of Medicine; 23: 463-483.

[26] Zou J, Yang Y, Liu Y. 2011. Release kinetics and cellular profiles for bFGF-loaded electrospun fibers: Effect of the conjugation density and molecular weight of heparin. Polymer; 52(15): 3357-3367.

[27] Hile DD, Amirpour ML, Akgerman A, Pishko MV. 2000. Active Growth Factor Delivery from Poly(-Lactide-Co-Glycolide) Foams Prepared in Supercritical CO_2. Journal of Controlled Release; 66: 177-185.

[28] Davies MJ, Mitchell CA, Maley MAL, Grounds MD, Harvey AR, Plant GW, Wood DJ, Hong Y, Chirila TV. 1997. *In vitro* Assessment of the Biological Activity of Basic Fibroblast Growth Factor Released from Various Polymers and Biomatrices. Journal of Biomaterials Applications; 12: 31-56.

[29] Cohen S, Yoshioka T, Lucarelli M, Hwang LH, Langer R. 1991. Controlled Delivery Systems for Proteins Based on Poly(Lactic Glycolic Acid) Microspheres. Pharmaceutical Research; 8: 713-720.

[30] Tabata Y, Takebayashi Y, Ueda T, Ikada Y. 1993. A Formulation Method Using D,L-Lactic Acid Oligomer for Protein Release with Reduced Initial Burst. Journal of Controlled Release; 23: 55-64.

[31] Tabata Y, Ikada Y. 1998. Protein Release from Gelatin Matrices. Advanced Drug Delivery Reviews; 31: 287-301.

[32] Tabata Y, Ikada Y. 1999. Vascularization Effect of Basic Fibroblast Growth Factor Released from Gelatin Hydrogels with Different Biodegradabilities. Biomaterials; 20: 2169-2175.

[33] Tabata Y. 2009. Biomaterial technology for tissue engineering applications. Journal of The Royal Society Interface; 6: S311-S324.
[34] Kawai K, Suzuki S, Tabata Y, Ikada Y, Nishimura Y. 2000. Accelerated Tissue Regeneration through Incorporation of Basic Fibroblast Growth Factor-Impregnated Gelatin Microspheres into Artificial Dermis. Biomaterials; 21: 489-499.
[35] Ward AG. 1954. The Physical Properties of Gelatin Solutions and Gels. British Journal of Applied Physics; 5: 85-90.
[36] Kusuhara H, Itani Y, Isogai N. 2011. Randomized controlled trial of the application of topical b-FGF-impregnated gelatin microspheres to improve tissue survival in subzone II fingertip amputations. Journal of Hand Surgery; 36E(6): 455-460.
[37] Tabata Y, Nagano A, Muniruzzaman M, Ikada Y. 1998. *In Vitro* Sorption and Desorption of Basic Fibroblast Growth Factor from Biodegradable Hydrogels. Biomaterials; 19: 1781-1789.
[38] Bin Choy Y, Cheng F., Choi H. 2008. Monodisperse gelatin microspheres as a drug delivery vehicle: Release profile and effect of crosslinking density. Macromolecular Bioscience; 8(8): 758-765.
[39] Bin Choy Y, Choi H, Kim K. 2009. Uniform Ethyl Cellulose Microspheres of Controlled Sizes and Polymer Viscosities and Their Drug-Release Profiles. Journal of Applied Polymer Science; 112(2): 850-857.
[40] Shipley GD, Ham RG. 1983. Control of Entry of Swiss-3t3 Cells into S-Phase by Fibroblast Growth-Factor under Serum-Free Conditions. Exp Cell Res; 146: 261-270.
[41] Richmond KMV, Kubler AM, Martin F, Jimenezdeasua L. 1980. The Stimulation of the Initiation of DNA-Synthesis by Fibroblast Growth-Factor in Swiss-3T3 Cells-Interactions with Hormones during the Pre-Replicative Phase. Journal of Cellular Physiology; 103: 77-85.
[42] Tabata Y, Ishii T, Muniruzzaman M, Hirano Y, Ikada Y. 2000. Influence of Gelatin Complexation on Cell Proliferation Activity and Proteolytic Resistance of Basic Fibroblast Growth Factor. Journal of Biomaterials Science - Polymer Ed; 11: 571-582.