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Research Paper

Heparin Immobilized Porous PLGA Microspheres for Angiogenic Growth Factor Delivery

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Purpose. Heparin immobilized porous poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres were prepared for sustained release of basic fibroblast growth factor (bFGF) to induce angiogenesis.
Materials and Methods. Porous PLGA microspheres having primary amine groups on the surface were prepared using an oil-in-water (O/W) single emulsion method using Pluronic F-127 as an extractable porogen. Heparin was surface immobilized via covalent conjugation. bFGF was loaded into the heparin functionalized (PLGA-heparin) microspheres by a simple dipping method. The bFGF loaded PLGA-heparin microspheres were tested for *in vitro* release and *in vivo* angiogenic activity.
Results. PLGA microspheres with an open-porous structure were formed. The amount of conjugated amine group onto the microspheres was 1.93 ± 0.01 nmol/mg-microspheres, while the amount of heparin was 95.8 pmol/mg-microspheres. PLGA-heparin microspheres released out bFGF in a more sustained manner with a smaller extent of initial burst than PLGA microspheres, indicating that surface immobilized heparin controlled the release rate of bFGF. Subcutaneous implantation of bFGF loaded PLGA-heparin microspheres in mice significantly induced the formation of new vascular microvessels.
Conclusions. PLGA microspheres with an open porous structure allowed significant amount of heparin immobilization and bFGF loading. bFGF loaded PLGA-HP microspheres showed sustained release profiles of bFGF *in vitro*, demonstrating reversible and specific binding of bFGF to immobilized heparin. They also induced local angiogenesis *in vivo* in an animal model.

KEY WORDS: angiogenesis; growth factor delivery; heparin; PLGA; porous microsphere.

INTRODUCTION

Angiogenesis, or neovascularization, is critical for the treatment of various vascular diseases, wound healing, and tissue regeneration. A lack of blood flow within a tissue may cause ischemia or delayed wound healing (1–3). Blood vessel formation also plays a pivotal role in regeneration of various tissues, since the supply of oxygen and nutrients must be sufficient to support growth and survival of cells. Polymeric delivery systems for inducing angiogenesis have been widely investigated. Various devices including microspheres, scaffolds, and hydrogels have been used for binding and releasing angiogenic growth factors, such as vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factor (aFGF and bFGF), and platelet-derived growth factor (PDGF) (4–9). In particular, biodegradable PLGA microspheres loaded with various growth factors have received much attention, because they can be directly injected into a tissue defect site to induce angiogenesis by local and sustained release actions (10). The most widely used method for preparing PLGA microspheres encapsulating protein drugs is a water-in-oil-in-water (W/O/W) double emulsion and solvent evaporation technique. However, the shear stress and presence of a water–oil interface during the formulation process adversely affect the structural integrity of proteins, resulting in denaturation and aggregation (11). It was previously reported that PLGA microspheres encapsulating various growth factors, although showing *in vivo* angiogenic activities to some extents, exhibited unpredictable and incomplete *in vitro* release profiles with very high initial bursts (12,13).

Many attempts have been made to enhance the stability of angiogenic growth factors. Heparin, a highly sulfated glycosaminoglycan, has binding affinities to various growth factors such as FGF, VEGF, heparin-binding epidermal growth factor (HBEGF), and transforming growth factor-β (TGF-β) (14). In the extracellular matrix, heparin is a primary binding site for growth factors until it is released to function in a cellular level. Heparin is also known to control the mitogenic activity of growth factors by regulating the growth of fibroblast and endothelial cells (6,14). Due to the specific interactions with various growth factors, heparin has been popularly used in fabrication of various bioactive implantable matrices such as collagen, alginate, and chitosan for controlled delivery of growth factors (15–17).

In this study, sustained release of bFGF was attempted by immobilization of heparin onto the surface of porous PLGA microspheres. Highly porous PLGA microspheres were first prepared by incorporation and subsequent leaching of a water

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soluble polymeric porogen during a single oil-in-water emulsion and solvent evaporation process (18). The resultant porous PLGA microspheres were used for derivatization with primary amine groups, to which heparin was conjugated using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). The heparin immobilized PLGA microspheres were characterized by quantifying the amounts of conjugated primary amine groups and heparin, and visualized with the use of a fluorescent probe. bFGF was loaded into the heparin immobilized microspheres by a simple dipping method. The release behaviors of bFGF from heparin modified/unmodified porous PLGA microspheres were comparatively examined *in vitro*, and their *in vivo* angiogenic potentials were also evaluated.

MATERIALS AND METHODS

Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA) (lactide/glycolide molar ratio: 75/25, average molecular weight: 10,000) and heparin sodium (Mw 12,000) was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Pluronic F127 ($(PEG)_{99}(PPG)_{69}(PEG)_{99}$) (average Mw 12,600) was obtained from BASF (Ludwigshafen, Germany). Recombinant human basic fibroblast growth factor (Mw 17.2 kDa, pI 9.0) was purchased from Peprotech (Seoul, Korea). Polyvinylalcohol (PVA; 88% hydrolyzed, Mw 13,000~23,000), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were from Sigma (St. Louis, MO). All other chemicals are of analytical grade.

Preparation of Porous PLGA Microspheres

Porous PLGA microspheres were prepared by using Pluronic F127 as an extractable porogen. A polymer mixture of 700 mg Pluronic F127 and 300 mg PLGA was dissolved in 3 ml of methylene chloride. The polymer solution was emulsified in 100 ml of 0.5% (w/v) PVA with a homogenizer at 1,500 rpm for 90 s. For solvent evaporation, the emulsion was placed in a hood under magnetic stirring condition. The resultant microspheres were centrifuged, washed three times with deionized water, and freeze-dried. Scanning electron microscopy (SEM) (Philips 535M, USA) was used for observing the morphology. Average sizes of microspheres and surface pores were determined by measuring at least 50 microspheres in the SEM image. Data are expressed as a mean ± SD.

Functionalization of PLGA Microspheres with Primary Amine Groups

Five hundred milligrams of porous PLGA microspheres was suspended in 10 ml of 0.1 M 2-morpholinoethanesulfonic acid (MES) buffer (pH 5.5) and hydrated for about 2 h. The PLGA polymer used in this study has an uncapped free carboxylic acid in one terminal end. To pre-activate the carboxylic groups, 95.9 mg EDC and 57.6 mg NHS were added to the solution, and then 50 μmol (3 mg) of ethylene diamine was slowly added. The reaction mixture was incubated overnight while stirring. The porous microspheres were separated and washed three times with deionized water, and freeze-dried. The amount of conjugated primary amine groups was determined by the fluorescamine assay. To determine the amount of the surface exposed amine groups, fluorescamine was reacted in aqueous condition. Nine milligrams of dried sample in 1 ml deionized water was added with 0.1 ml of 5 mg/ml fluorescamine in acetone and mixed well by vortexing. Samples were dried under vacuum, dissolved in 1 ml acetone, and centrifuged at 12,000 rpm for 10 min. The supernatant was detected for fluorescence at wavelengths of 390 nm for excitation and 475 nm for emission (Shimadzu RF-5301PC, Japan). The amount of primary amine groups was determined based on a calibration curve constructed from poly(ethylene glycol)-bis-amine.

Visualization of Amine Functionalized PLGA Microspheres

Amine functionalized PLGA microspheres were also visualized by labeling with fluorescein isothiocyanate. Forty milligrams of microspheres was pre-hydrated in 3 ml phosphate buffer saline (PBS) solution (pH 7.4) overnight, and then exchanged to 3 ml 0.1 M sodium carbonate (pH 9). After 4 h incubation, 7 mg (10 × molar ratio of conjugated amine) of fluorescein isothiocyanate (FITC isomer I, Mw 389.4) was added and incubated at 4°C overnight. After

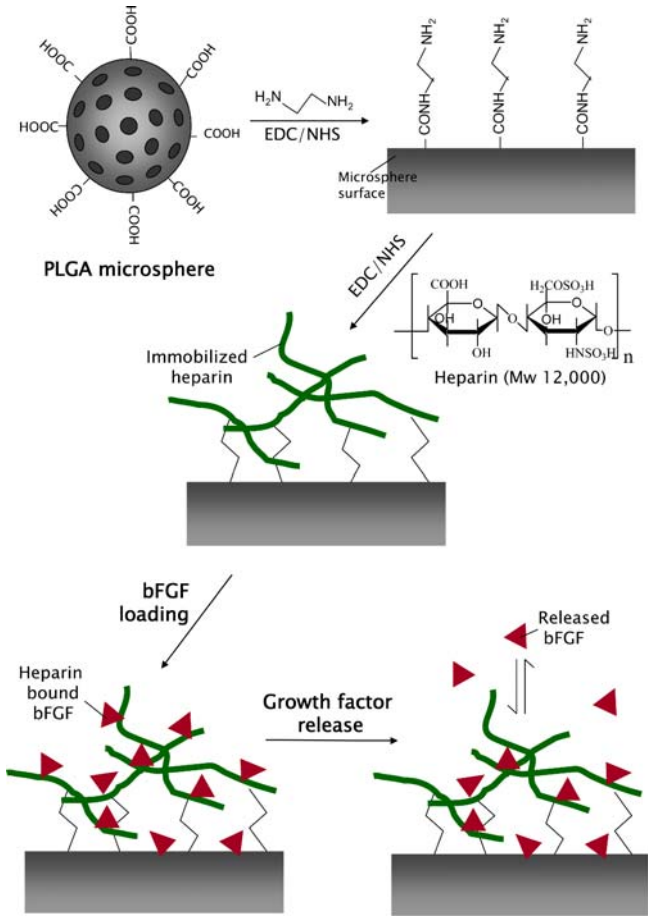

Fig. 1. Illustration of procedures for angiogenic growth factor delivery by heparin immobilized microspheres.

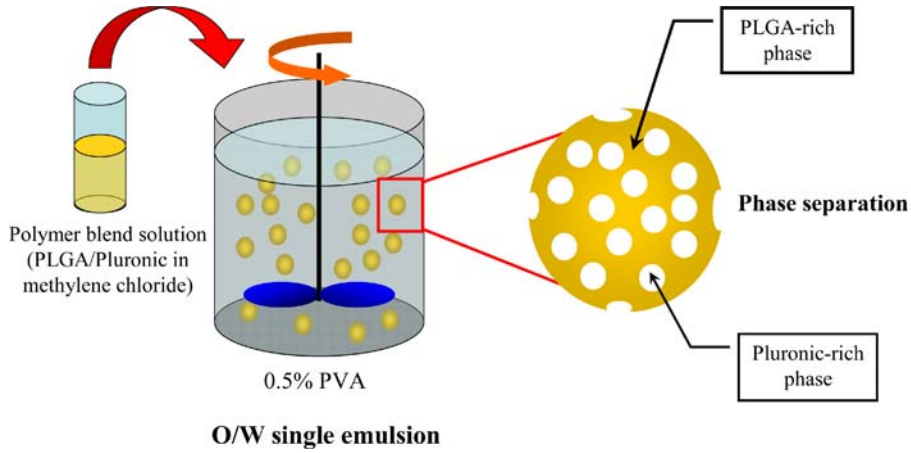

Fig. 2. Fabrication process of porous PLGA microspheres based on an oil-in-water single emulsion and solvent evaporation method.

removing free FITC by washing four times with deionized water and the FITC-labeled microspheres were freeze-dried. The FITC labeled samples were observed under a confocal laser scanning microscope (Carl Zeiss LSM510, Germany).

Heparin Immobilization

Amine functionalized microspheres (250 mg) were suspended in 0.1 M MES buffer (pH 5.5) and hydrated for 2 h, to which 47.9 mg EDC and 28.8 mg NHS were added, and then 4.8 μmol of heparin (1.93 mg, 10 × molar ratio of conjugated amine groups) was slowly added. The reaction mixture was incubated overnight while rotating. Samples were collected by centrifugation and freeze-dried. The amount of immobilized heparin was examined by a toluidine blue assay. Ten milligrams of dried heparin conjugated microspheres (PLGA-heparin) was suspended in 1 ml of deionized water. After hydrating overnight, 1 ml of 0.005% toluidine blue solution containing 0.2% NaCl and 0.01 N HCl was added to the sample and incubated at room temperature for 3 h. To the solution, 1 ml of *n*-hexane was added, mixed vigorously by vortexing, and centrifuged at 2,500 rpm for 5 min. After removing *n*-hexane, absorbance at 631 nm was detected with a spectrophotometer (Shimadzu UV-1601, Japan). The calibration curve was constructed based on using a series of heparin concentrations.

Visualization of Heparin Immobilized Microspheres

Heparin immobilization was also visualized by conjugating rhodamine labeled heparin. Fifty milligrams of heparin was dissolved in 2 ml of dimethylformamide at 50°C, then activated by adding 33 mg of *p*-nitrophenyl chloroformate (TCI, Tokyo, Japan) and 26.3 mg pyridine at 0°C and reacted under nitrogen atmosphere at room temperature for 2 h. The clear part was separated and added with 5 mg of rhodamine B amine and 8.4 mg triethylamine, and reacted overnight under nitrogen condition at room temperature. Samples were dialyzed to remove the unreacted rhodamine, and then freeze-dried. The resultant rhodamine labeled heparin was used to conjugate to the amine functionalized microspheres.

In Vitro Release of bFGF

bFGF was loaded within heparin conjugated or unconjugated microspheres by a solution dipping method. Forty-five milligrams of heparin conjugated microspheres were suspended in 2 ml of PBS containing 50 μg bFGF overnight

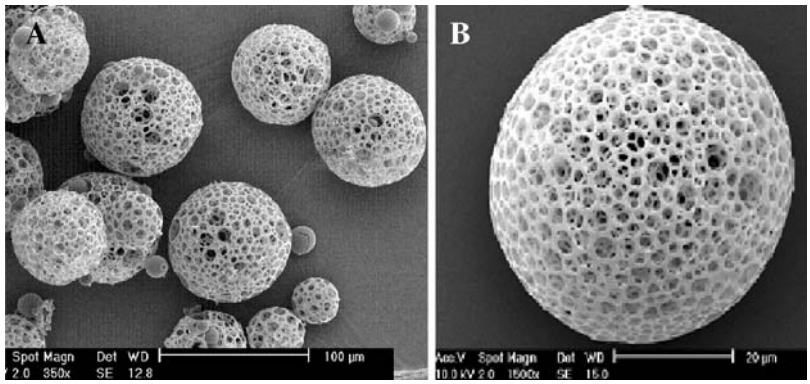

Fig. 3. Morphology of porous PLGA microspheres observed by scanning electron microscopy (SEM). (A) 350×, (B) 1,000× magnification.

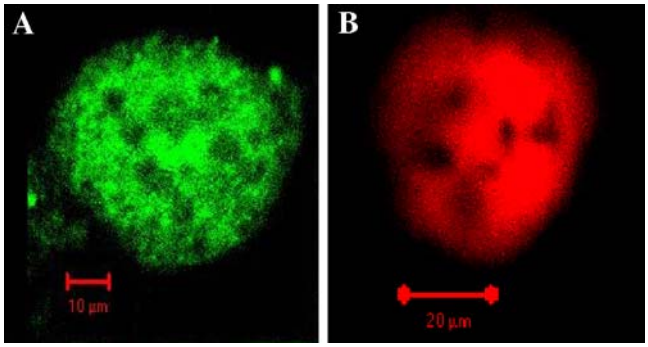

Fig. 4. Confocal laser scanning microscopy of surface modified microspheres by labeling (A) amine groups with FITC and (B) heparin with rhodamine.

at 4°C in a rotating condition. bFGF loaded microspheres were recovered by centrifugation, washed with PBS to remove free bFGF, and lyophilized. No cryoprotectants were used for the lyophilization. Fifteen milligrams of bFGF loaded microspheres was suspended in 2 ml of PBS (1 mg/ml bovine serum albumin, 0.02% sodium azide) at 37°C. At fixed time intervals, the supernatants were collected by centrifugation, and the amount of the released bFGF was determined by using the Quantikine® Immunoassay kit (R&D Systems, Minneapolis, MN).

In Vivo Analysis of Angiogenesis

Heparin immobilized microspheres loaded with bFGF were injected subcutaneously onto the dorsal side of BALB/c nude mouse (Samtako, Kyunggido, Korea) using a 1 ml syringe with a 24G needle. A total of six mice were divided into two groups, one for the blank microspheres as a control, and the other for the growth factor loaded microspheres. The left side was injected with PLGA microspheres and the right side with PLGA-heparin microspheres. Ten mg of microspheres suspended in 0.5 ml of 0.9% NaCl solution was administered per injection. After 1 week, mice were sacrificed and dissected to obtain skin tissues at the region of injection. Photographs were taken right after dissection, and the tissues were fixed in 10% (v/v) formalin solution at 4°C overnight, and then exchanged to phosphate buffer saline for further storage. The tissues were dehydrated by processing in ethanol solutions (70, 80, 90, 95, and 100 v/v%) and xylene, embedded in paraffin, and sectioned with a microtome (Leica RM 2125RT, Germany) at a width of 8 μm. The direction of sectioning was perpendicular to the skin layer and transverse to the mouse body, which was roughly perpendicular to the lining of capillaries and cross-sections of blood vessels could be observed. Sectioned tissues were stained with Harris hematoxylin and eosin, to be observed and photographed using a microscope (Carl Zeiss, Germany) equipped with a camera (Jenoptik, Jena, Germany). Capillary density (no. of capillaries per square centimeter) was analyzed with the Image J software (NIH, Bethesda, MD), and was determined as the total number of capillaries divided by total area of the subcutaneous region at the site of implant. Animal studies were performed according to the "Principles of Laboratory Animal Care" guided by the National Institute of Health.

RESULTS AND DISCUSSION

Figure 1 illustrates the procedures for surface functionalization of porous PLGA microspheres with primary amine groups and subsequent heparin immobilization, and followed by growth factor release. The PLGA polymer used in this study had an uncapped free carboxylic acid at one terminal

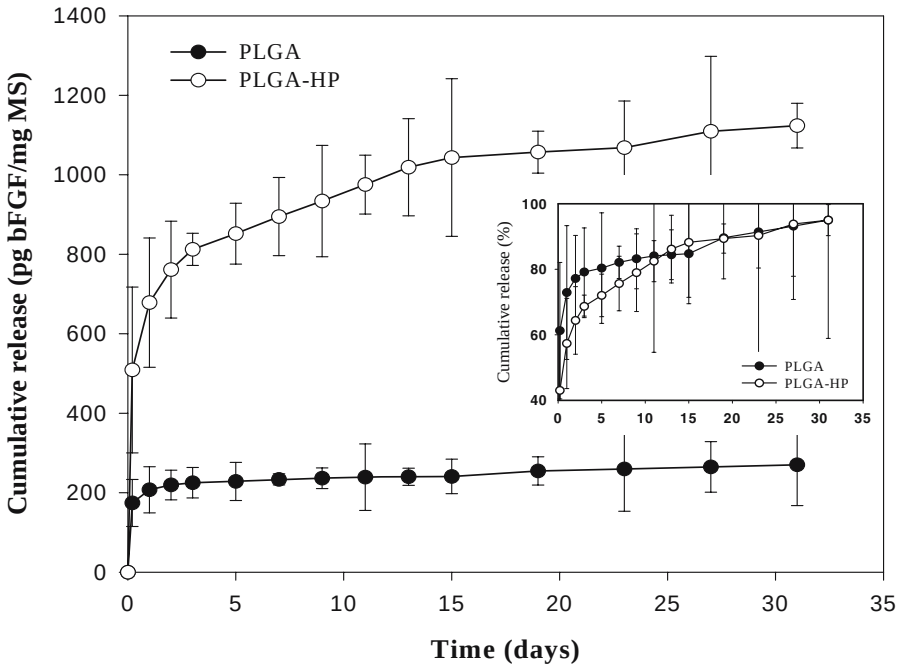

Fig. 5. *In vitro* release profiles of bFGF in absolute amount (pg bFGF/mg MS), insert shows cumulative release in percent (%). Data are expressed as a mean ± SD ($n = 3$).

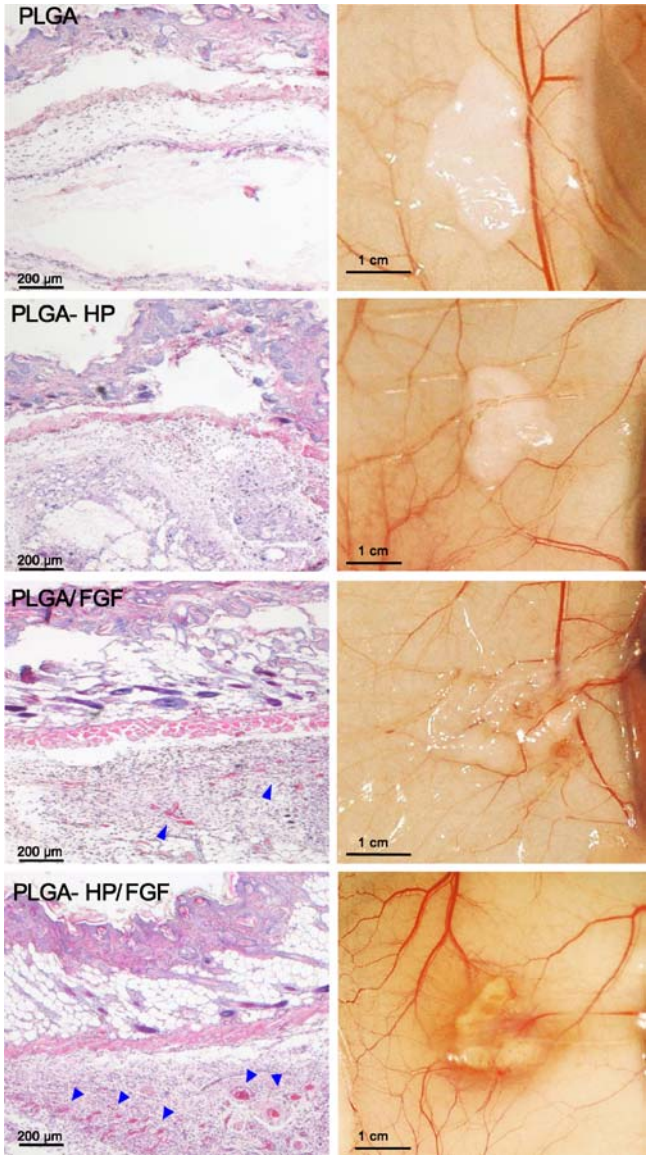

Fig. 6. Photographs of dissected tissues (*right column*) and histochemical staining results (*left column*) 1 week after injection of PLGA, PLGA-HP, bFGF loaded PLGA, and bFGF loaded PLGA-HP microspheres (*first*, *second*, *third*, and *fourth* row, respectively).

end, which was expected to be preferentially oriented towards the aqueous phase when porous PLGA microspheres were hydrated. The surface exposed carboxylic acids were utilized to produce surface amine groups that were conjugated with heparin via EDC/NHS chemistry (19–21). Porous PLGA microspheres were prepared by a previously reported method using Pluronic F127 as an extractable porogen in an oil-in-water single emulsion and solvent evaporation process (18). As shown in Fig. 2, the polymer blend mixture co-dissolved in an organic droplet phase is separated into a PLGA rich phase and a Pluronic rich phase as solvent exchange between water and methylene chloride takes place. During solvent evaporation, the leaching out of Pluronic F127 from the emulsion droplets gives rise to a porous structure. Figure 3 shows the morphology of the porous PLGA microspheres observed by SEM. The microspheres had an average diameter of 50.8 ± 8.4 μm with an average pore diameter of approximately 4.8 ± 1.9 μm.

Heparin was immobilized onto the PLGA surface by conjugation onto amine groups projecting on the surface of the PLGA matrix (22–24). By hydrating the microspheres in buffer solution, the terminal carboxylic acid group of PLGA was surface exposed, activated with EDC and NHS, and then conjugated with ethylenediamine via an amide linkage (19–21). The amount of conjugated amine groups on the PLGA surface was 1.93 ± 0.01 nmol/mg-microspheres, as determined by the fluorescamine assay. The amount of immobilized heparin as determined by the toluidine blue assay (25) was 95.8 pmol/mg-microspheres. Figure 4-(A) shows the surface amine groups on the PLGA microspheres visualized by labeling with FITC, as observed by confocal laser scanning microscopy (26–28). Green fluorescence can be detected throughout the polymeric matrix of the microspheres, demonstrating the uniform distribution of conjugated amine groups. The dark spots are indicative of the open pores. Immobilized heparin was also visualized by using rhodamine as a probe. Figure 4-(B) shows the red fluorescence image observed by confocal microscopy, which is similar to that of FITC labeled amine. Rhodamine labeled heparin is evenly distributed throughout the whole microsphere, in which dark spots reside due to the porous structure.

bFGF was loaded within heparin immobilized and unimmobilized PLGA microspheres by a simple dipping method. The loading amount of bFGF for PLGA-heparin (HP) microspheres was 1,124 ± 103 pg per mg of dry microspheres, whereas that for PLGA microspheres was 270 ± 56 pg per mg of dry microspheres. The greatly enhanced loading of bFGF for PLGA-HP microspheres was certainly due to the fact that bFGF can be tightly bound to heparin moieties immobilized on the surface. Specific interaction between bFGF and heparin played a key role in increasing the loading amount of bFGF for the PLGA-HP microspheres. Figure 5 shows release profiles of bFGF from porous PLGA-HP and PLGA microspheres. PLGA-HP microspheres released out bFGF in a more sustained manner with reduced extent of initial burst than PLGA microspheres. For PLGA-HP microspheres, bFGF

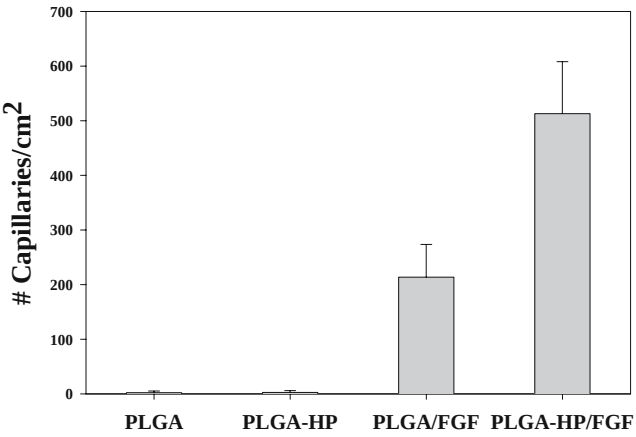

Fig. 7. Capillary density calculated from sectioned tissue samples 1 week after injection of PLGA, PLGA-HP, bFGF loaded PLGA (PLGA/FGF), and bFGF loaded PLGA-HP (PLGA-HP/FGF) microspheres. Data are expressed as a mean ± SD (n = 3).

was released out over a 30-day period, although the initial burst release after 1-h incubation was about 40%. In contrast, PLGA microspheres exhibited an immediate release of about 60% at the first hour upon incubation. The observed sustained bFGF release profile from PLGA-HP microspheres was likely dictated by specific binding of bFGF to heparin moieties immobilized on the surface (29). The dissociation rate of bFGF from the bFGF-heparin complex in the PLGA-HP microspheres is probably governed mainly by a thermodynamic equilibrium between free bFGF in the release medium and unreleased bFGF bound to heparin in the microspheres. Hence the sustained release pattern of bFGF from PLGA-HP microspheres was mainly controlled by the amount of released and free bFGF species in the medium, not by a diffusion controlled mechanism. In this sense, the experimental conditions for bFGF release would be very important in determining the overall release profile. For instance, the amount of PLGA-HP microspheres and the volume of buffer medium could alter the release pattern by changing the dissociation rate of bound bFGF depending on the amount of free bFGF species in the release medium. Therefore, heparin immobilization on the surface of porous PLGA microspheres not only provided an opportunity for enhancing the loading amount of bFGF, but also improved the sustained release profile with reduced initial burst. The observed initial burst for PLGA-HP microspheres was likely caused by nonspecifically and weakly bound bFGF species on the surface that were immediately desorbed upon incubation. Similar sustained release patterns were observed for heparin conjugated hydrogel delivery systems (7,14), but the current delivery strategy based on porous and injectable microspheres with heparin modification has not been reported.

The angiogenic potential of the bFGF releasing PLGA-HP microspheres was examined using an animal model. Figure 6 (right column) shows the photographs of tissue specimens during dissection. As shown, the injected microspheres were trapped in capsules of fibrous tissue and remained at the site of injection. The fibrous capsules for blank microspheres remained uninvaded by blood vessels. On the other hand, bFGF loaded PLGA-HP microspheres were covered and surrounded by a network of newly formed blood vessels, a sign of angiogenesis. The results were particularly dramatic for PLGA-heparin microspheres, while PLGA microspheres gave just moderate results. The histochemical staining results for the corresponding tissue are shown at the left column of Fig. 6. The injected microspheres were not clearly visible, probably because they were partially dissolved out during the specimen processing step using ethanol and xylene for dehydration. However, the observed fibrous tissue was used as a spatial indicator of the injection site for the PLGA-heparin microspheres to examine the formation of new blood vessels in the vicinity. The fibrous tissue was formed as a layer beneath the dermis by infiltration of neutrophils and macrophages (densely stained with hematoxylin) (30). Similar to the results above, fibrous tissue for blank microspheres was uninvaded by blood vessels. For the growth factor loaded microspheres, however, newly formed blood capillaries can be seen by the round cross-sections filled with red blood cells. PLGA-HP microspheres gave rise to much more blood vessels, as well as larger sizes. These results were quantified by calculating the capillary density (Fig. 7), that is, the total number of capillaries divided by total area of the subcutaneous region at the site of implant. The boundaries of total area were determined to include the subcutaneous layer which covers or lies beneath the space of implant along with the surrounding fibrous capsule. Capillary density for microspheres without bFGF was extremely low, but they were greatly enhanced for the bFGF loaded PLGA-HP microspheres ($513 \pm 95/cm^2$). The sustained release pattern of bFGF from PLGA-HP microspheres observed *in vitro* was clearly responsible for the angiogenic activity in the animal study. The bFGF release amount and duration, however, should be optimized to maximize the formation of new blood vessels in the tissue. These results, along with the release data, revealed that heparin immobilized PLGA microspheres were capable of reversible, specific binding of bFGF, which was released in a controlled manner when administered by injection.

CONCLUSIONS

A local and sustained release formulation for angiogenic growth factor delivery was developed. Porous PLGA microspheres were fabricated by an oil-in-water single emulsion and solvent evaporation method with the use of Pluronic F127 as a porogen. The microspheres were functionalized with primary amine groups, followed by immobilization of heparin. bFGF was loaded into PLGA or PLGA-HP microspheres by a solution dipping method. PLGA-HP microspheres showed sustained release profiles of bFGF *in vitro*. Additionally, PLGA-HP microspheres greatly induced local angiogenesis *in vivo*.

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