Pharmaceutical Research, Vol. 25, No. 10, October 2008 (© 2008)
DOI: 10.1007/s11095-008-9685-1

Research Paper

In Vitro and *In Vivo* Release of Vascular Endothelial Growth Factor from Gelatin Microparticles and Biodegradable Composite Scaffolds

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Received January 15, 2008; accepted March 24, 2008; published online July 29, 2008

Purpose. This work evaluated gelatin microparticles and biodegradable composite scaffolds for the controlled release of vascular endothelial growth factor (VEGF) *in vitro* and *in vivo*.
Methods. Gelatin crosslinking, VEGF dose, and buffer type were investigated for their effects on VEGF release. Release was also evaluated from microparticles confined within porous polymer scaffolds (composites). *In vitro* and *in vivo* studies were conducted using radiolabeled VEGF.
Results. The effect of VEGF dose on its fractional release from gelatin microparticles *in vitro* was minimal, but the addition of collagenase to the buffer resulted in a higher cumulative release of VEGF. Gelatin crosslinking extent was a significant factor on release from both microparticles alone and composite scaffolds *in vitro* and *in vivo*. VEGF bioactivity from composite scaffolds *in vitro* was maintained above 90% of the expected bioactivity over 14 days.
Conclusions. VEGF release kinetics were dependent on the extent of gelatin crosslinking and were characteristic of the specific growth factor due to the effects of growth factor size, charge, and conformation on its complexation with gelatin. These studies demonstrate the utility of gelatin microparticles and their composite scaffolds as delivery vehicles for the controlled release of VEGF for tissue engineering applications.

KEY WORDS: bone tissue engineering; controlled drug delivery; gelatin microparticles; porous polymer scaffold; vascular endothelial growth factor.

INTRODUCTION

Angiogenesis, the formation of new blood vessels, is an important event in several biological processes and especially in the wound healing environment. Injury sites are characterized by high metabolic activity and hypoxia, requiring a dense capillary network during repair to deliver oxygen and nutrients and clear away cell debris (1,2). Bone, in particular, is a highly vascularized tissue and angiogenesis is crucial for bone regeneration (3–6). Thus, it is vital to address the issue of angiogenesis in strategies for bone regeneration.

In order to mimic the normal temporal pattern of bone healing, controlled delivery of growth factors can be used to induce angiogenesis early on followed by osteogenesis. Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor that has often been used to stimulate blood vessel formation in a number of studies (7–10) and has also been shown to promote the formation of mineralized tissue (11). However, it is usually delivered via a carrier because VEGF has a clearance half-life of less than 1 h following injection *in vivo* (12). Therefore, controlled release is required in order to mimic the temporal presence of VEGF in the bone wound healing environment.

Our laboratory is developing a system of novel biomaterials for the controlled delivery of bioactive molecules. This system is based on gelatin microparticles which serve as delivery vehicles for the growth factors. Gelatin is commonly used for several tissue engineering strategies and in various forms, including as disks and microparticles, for the delivery of growth factors such as basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF-β1), insulin-like growth factor-1, and bone morphogenetic protein-2 (BMP-2) (13,14). Controlled release is based on electrostatic attractions between the growth factor and the gelatin; depending on the gelatin type (acidic or basic), it can be either negatively or positively charged at physiological pH. Therefore, for VEGF with an isoelectric point (IEP) of 8.6, acidic gelatin (IEP=5) was chosen as the best carrier. The mechanism of release is based on the degradation of the gelatin; the enzymatic degradation of gelatin occurs by matrix metalloproteinases such as collagenase, and the extent of

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ABBREVIATIONS: bFGF, basic fibroblast growth factor; BMP-2, bone morphogenetic protein-2; Coll, collagenase-containing phosphate buffered saline; GPC, gel permeation chromatography; HUVECs, human umbilical vein endothelial cells; IGF-1, insulin-like growth factor-1; IEP, isoelectric point; microCT, microcomputed tomography; PBS, phosphate buffered saline; PPF, poly(propylene fumarate); SEM, scanning electron microscopy; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor; VOI, volume of interest.

gelatin crosslinking can affect this degradation. Different release profiles have been achieved by varying crosslinking extents and thus the rate of degradation (15). In our delivery system, composites scaffolds are generated which consist of these gelatin microparticles incorporated within the porous network of a polymer scaffold. The polymer scaffold, made of poly(propylene fumarate) (PPF), acts to confine microparticles at the defect site and can help maintain structural integrity during healing in addition to being biodegradable and biocompatible (16).

The goal of this study was to evaluate acidic gelatin microparticles for the controlled release of VEGF. First, we investigated the effects of gelatin crosslinking, growth factor dose, and release medium on VEGF release kinetics. *In vitro* release studies of VEGF from gelatin microparticles were conducted and crosslinking extent, growth factor dose, and buffer type were varied as parameters. After identifying which of these parameters affected VEGF release from microparticles alone, we tested these parameters for *in vitro* release from composite scaffolds of gelatin microparticles within a porous PPF scaffold. An *in vivo* study was also conducted with optimized parameters to evaluate the release profile of VEGF from the composite scaffolds in a mouse model. Finally, a bioactivity study was performed to test the angiogenic potential of VEGF released from the composite scaffolds.

MATERIALS AND METHODS

Experimental Design

Acidic gelatin (IEP=5) was evaluated for controlled release of VEGF (IEP=8.6). Gelatin microparticles were crosslinked with two concentrations of glutaraldehyde (10 and 40 mM) and a low and high dose of growth factor (6 and 60 ng of VEGF per mg of dry microparticles) was used for loading. Samples were incubated in two buffer types: phosphate buffered saline (PBS) and collagenase-containing PBS (Coll) with 400 ng/mL of bacterial collagenase 1A—a collagenase which recognizes and digests part of gelatin's amino acid sequence (17).

Gelatin Microparticle Preparation

Five grams of gelatin (Nitta Gelatin Co., Osaka, Japan) were dissolved in 45 mL of water and added dropwise to 200 mL olive oil to create a water-in-oil emulsion (18). The solution was stirred at 500 rpm and chilled to 10°C for 1.5 h; microparticles were then collected by washing with acetone and vacuum filtration. They were crosslinked overnight in a glutaraldehyde solution and the reaction was terminated by the addition of glycine (25 mg/mL) to block residual aldehyde groups. The microparticles were again washed in acetone and collected by filtration, lyophilized, and then sieved to obtain particles ranging from 50–100 μm.

VEGF incorporation was achieved by diffusional loading; a solution of growth factor in PBS was dripped onto the microparticles at a volume of 5 μL per mg of dry microparticles (18). Following vortexing, the loaded microparticles were incubated at 4°C for 20 h. The VEGF solution consisted of a mix of radiolabeled and unlabeled growth factor in mass ratio of 0.06:1 for the low dose and 0.01:1 for the high dose (to minimize the use of radioactive material and exposure). Radiolabeled VEGF (radioiodinated with ^{125}I using a modification of the iodogen method (19)) was obtained from PerkinElmer (Waltham, MA) while cold VEGF was obtained from Peprotech (Rocky Hill, NJ) for the *in vitro* studies and R&D Systems (Minneapolis, MN) for the *in vivo* study.

Microparticle Swelling

Swelling studies were conducted with $n=5$ samples per group using microparticles crosslinked with 0, 10, and 40 mM glutaraldehyde (18). Briefly, 50 mg of microparticles were swollen in excess PBS overnight, patted dry to remove surface water and weighed (W_S), lyophilized, and weighed again (W_D). Fold swelling ratios (S), which provide an indication of the water uptake per gram of microparticle, were calculated as:

$$S = \frac{(W_S - W_D)}{W_D}$$

PPF Synthesis

PPF synthesis involved the generation of a diester intermediate followed by polymerization (20). First, diethyl fumarate, propylene glycol, hydroquinone, and zinc chloride were combined in a 1:3:0.003:0.01 molar ratio and stirred at 300 rpm and heated to 130°C under a nitrogen purge. Ethanol was distilled out and the reaction was stopped when 90% of the theoretical yield of ethanol is removed. The temperature was then set to 100°C and vacuum (<1 mmHg) was applied. Every 30 min, the temperature was raised 10°C to 130°C and maintained while propylene glycol was removed as a distillate. Samples were collected every hour for gel permeation chromatography (GPC) analysis and the reaction was terminated once the desired molecular weight was reached. Purification was achieved through a 1.85% hydrochloric acid wash followed by a series of aqueous washes to remove zinc chloride and an ether wash to remove hydroquinone. The purified polymer was then vacuum dried to eliminate any residual solvent and evaluated for final molecular weight by GPC.

Porous PPF Scaffold Fabrication

To generate porous polymer scaffolds, PPF and N-vinyl pyrrolidone were mixed together in a 1:1 mass ratio; this was followed by addition of 0.5 wt.% benzoyl peroxide (0.1 mg/mL in acetone) and 80 wt.% NaCl (300–500 μm crystals) (21). This paste was packed into molds (7.5 mm diameter, 1 mm height) and crosslinked overnight at 60°C. The scaffolds were leached in water for 3 days to remove the salt, resulting in a porous structure. These porous PPF scaffolds were then lyophilized overnight, and the surface areas were sanded down to achieve a height of 1 mm. Following flushing with 70% ethanol, the scaffolds were again lyophilized overnight.

Composite Scaffold Generation

Composite scaffolds consisted of gelatin microparticles entrapped within the pores of the PPF scaffolds. 2.5 mg each

of loaded and unloaded gelatin microparticles were mixed together in 30 μL of a 24% (w/v) solution of Pluronic F-127 (Sigma, St. Louis, MO) in water, injected into a porous PPF scaffold, and allowed to gel at room temperature for 10 min (22). Depending on the experimental group, the loaded microparticles consisted of either the 10 mM or 40 mM type swollen with VEGF. The unloaded microparticles were 40 mM basic gelatin microparticles (swollen with PBS alone) that were used to simulate BMP-2 loading for use of the scaffolds as dual growth factor delivery systems (23).

Microcomputed Tomography (microCT)

MicroCT analysis provided a means of quantitatively measuring the 3D porosity and porous interconnectivity of the PPF scaffolds in a nondestructive manner. Six 7.5×1 mm cylindrical scaffolds were scanned with a SkyScan 1172 high-resolution microCT imaging system (Aartselaar, Belgium) at a 7 μm resolution with a voltage of 40 kV and current of 240 μA. Volumetric reconstruction and analysis were conducted using Nrecon and CT-analyser software provided by SkyScan. A global threshold of 50–255 was used for all analyses.

Porosity was calculated by drawing a cylindrical volume of interest (VOI) within the scaffold and measuring the percent of binarized object volume (the actual polymer volume of each scaffold) within this VOI. The percent porosity was calculated as 100% − percent binarized object volume.

Interconnectivity within the scaffold was defined as the percentage of porous volume (V_P, volume of void space within the scaffold) that is accessible by a sphere with a given diameter (set to range as multiples of the resolution size from 28–196 μm). A VOI larger than the scaffold was drawn and a sphere diameter was set. A shrinkwrap function was used to shrink this VOI through any openings which the sphere could pass, and a measurement of the VOI (V) and volume of the binarized object (scaffold) (V_S) were taken. If 100% of the porosity was accessible to the sphere, then $V=V_S$; otherwise, $V_S<V$ because the volume of the VOI includes the volume of the scaffold plus any void space that is not accessible. Interconnectivity was calculated as follows:

$$\text{Interconnectivity} = \frac{V_P - (V - V_S)}{V_P} * 100\%$$

The porous volume can be calculated for each scaffold from the V_S and the percent binarized object volume obtained from the previous porosity measurements:

$$V_P = \frac{V_S}{\%\ \text{binarized object volume}/100\%} - V_S$$

Scanning Electron Microscopy

Samples were freeze-dried and mounted on aluminum stages, sputter-coated with gold for 1 min, and observed by scanning electron microscopy (SEM, FEI Quanta 400 Environmental, Hillsboro, OR) at an accelerating voltage of 20 kV. Microparticles were observed as is, while scaffolds were sliced in half in order to view both surface areas and cross-sections.

In Vitro Release

Growth factor loading of the microparticles and generation of the composite scaffolds was achieved as described above. Scaffolds or microparticles alone were incubated in buffer at 37°C and agitated at 70 rpm. At each time point ($n=6$), the buffer was removed and replaced with fresh buffer. For microparticle release studies, the samples were centrifuged at 3,000 rpm for 5 min before each time point to reduce loss of microparticles during buffer removal. Standards with known amounts of radiolabeled growth factor were used to account for radioactive decay. Release was quantified by monitoring the radioactivity in the removed buffer using a gamma counter (Cobra II Autogamma, Packard, Meridian, CT) and the results were correlated to a standard curve.

Percent cumulative release was determined by normalizing total VEGF released by each time point with the total amount incorporated within the scaffolds or microparticles (this being the sum of VEGF released over the study with the amount remaining in the scaffolds or microparticles at the last time point). Release rates were calculated as the slope of percent cumulative release over the stated time period and are given as change in percent cumulative release per day.

In Vivo Release

This study was in compliance with the appropriate institutional animal care and use committee at Kyoto University. Microparticles, PPF scaffolds, and dry Pluronic were sterilized by ethylene oxide. Water and PBS were syringe-filtered with 0.22 μm filters. Composite scaffolds were measured for initial radioactivity and then implanted into the dorsal subcutis of 6 week old female ddY mice (Shimizu Laboratory Supply Inc., Japan) along the median, approximately 15 mm away from their tail root. At each time point ($n=3$), the skin around the implanted site was excised and the underlying fascia was thoroughly wiped to absorb radiolabeled VEGF, followed by retrieval of the composite scaffold. The radioactivity remaining within scaffold, the removed skin, and the filter paper were measured on a gamma counter (ARC-301B, Aloka Co., Japan). Data are shown as percent of radioactivity released as determined by subtracting initial radioactivity by that remaining at each time point (accounting for decay). Time points were at 1, 3, 7, and 14 days.

VEGF Bioactivity

An *in vitro* cell-based assay was used to determine the biological activity of VEGF released from composite scaffolds. Human umbilical vein endothelial cells (HUVECs) (ATCC, Manassas, VA) were used with basal medium (Cell Systems, Kirkland, WA) containing 10% fetal bovine serum and 50 μg/mL gentamicin. Composite scaffolds with 10 and 40 mM microparticles, loaded with a high dose of VEGF (60 ng per mg of dry microparticles), were synthesized as described above and placed in a 24 well plate with 1 mL of media/well. For each time point ($n=3$), media were collected off the scaffolds and replaced with fresh media; the VEGF amounts in these media were calculated using the release kinetics obtained from the *in vitro* release study described

above. As in Peters *et al.* (24), HUVECs were seeded 24 h before each time point in 24-well plates at 9,500 cells/well and incubated with media containing 20 ng/mL VEGF to increase plating efficiency. After 24 h, the plating media were removed off the cells and replaced with the collected media from the scaffolds, or with controls consisting of media containing soluble VEGF at 0–40 ng/mL concentrations. The HUVECs were incubated for 48 h, trypsinized, and counted on a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA). Time points were at 1, 3, 7, and 14 days. The growth factor bioactivity was determined by comparing the stimulatory effect of released VEGF from composites (the observed increase in cell growth) to control VEGF (cell growth due to equivalent amounts of VEGF added directly to culture medium).

Statistics

Main effects of gelatin crosslinking, growth factor dose, and buffer type were evaluated using a regression model with release as a response-dependent variable ($p<0.05$) on SAS statistical software (Cary, NC). Release rates and bioactivity were analyzed using a multi-factor analysis of variance followed by a Tukey–Kramer multiple comparisons test ($p<0.05$) to determine statistical significance. The results are reported as mean ± standard deviation for $n=6$ for the *in vitro* studies and $n=3$ for the *in vivo* studies.

RESULTS

Microparticle Swelling

The fold swelling ratios of the microparticles crosslinked with 0, 10, and 40 mM glutaraldehyde were 20.8±0.7, 12.5±0.3, and 9.1±0.2, respectively. This ratio is an indication of how much water can be absorbed per gram of microparticle. With increasing crosslinking extents, the swelling ratio decreased significantly ($p<0.05$), revealing that water uptake also is decreasing.

SEM Analysis

Gelatin microparticles were observed via SEM, confirming their spherical shapes and sizes (Fig. 1a). Figure 1b shows the porous structure of the scaffolds and the incorporation of the microparticles within the Pluronic gel in the pores of the scaffolds.

Porous PPF Scaffolds

GPC analysis determined the final number average molecular weight of the PPF to be 1770 with a polydispersity index of 1.7. The porosity of the PPF scaffolds as measured by microCT analysis averaged 70.9±1.1% (see Fig. 2 for a representative microCT scan of the scaffolds). The interconnectivity, given as the percent of porosity which is accessible by a sphere of a given diameter, was evaluated over a range of diameters (24, 56, 84, 112, 140, 168, 196 μm). The accessible porosity of the scaffolds was consistently at 98% for diameters up to 140 μm and then dropped drastically to 75% at 168 μm, indicating that the size of the interconnections between pores lies between 140–168 μm.

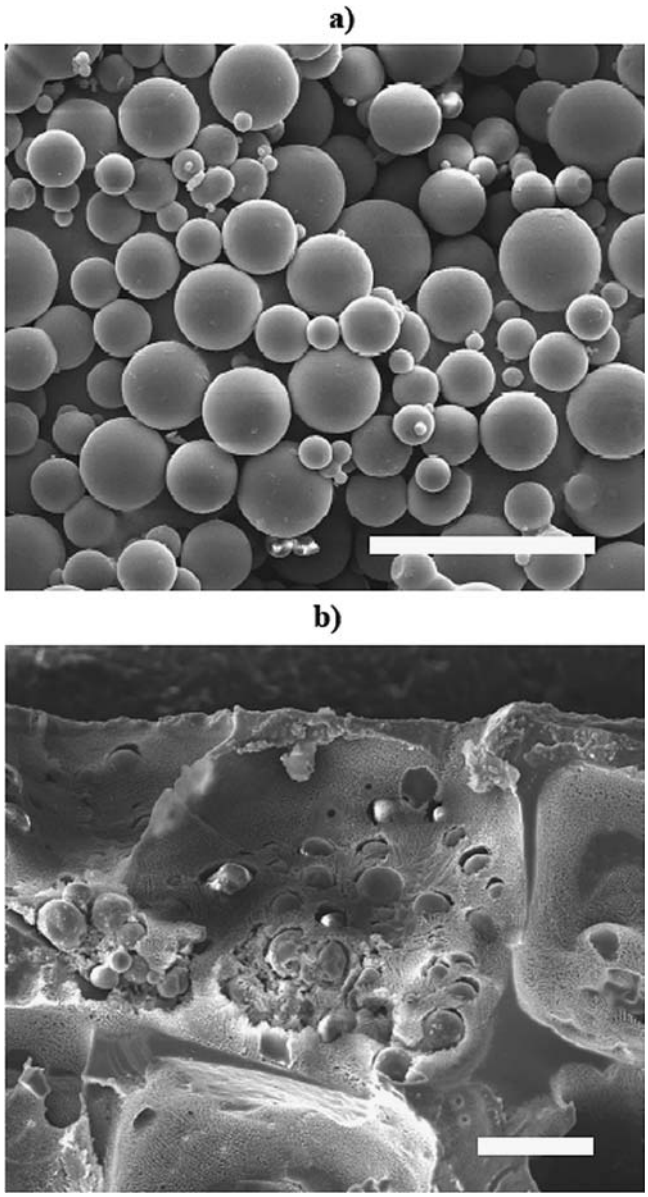

Fig. 1. SEM analysis of gelatin and PPF scaffolds. **a**) 10 mM acidic gelatin microparticles and **b**) cross-section of a composite scaffold with microparticles embedded in a Pluronic gel within the porous structure of a PPF scaffold. Bar represents 100 μm.

Release from Gelatin Microparticles

VEGF release from acidic gelatin microparticles was first evaluated *in vitro* (Fig. 3). The crosslinking of the gelatin (10 mM and 40 mM) and the dose of the growth factor were varied (6 and 60 ng per mg of dry microparticles as low and high doses). Buffers of PBS and collagenase-containing PBS (Coll) were also used. The main effects of gelatin crosslinking, growth factor dose, and buffer type on overall release are given in Table I. To quantify effects between formulations, the release profiles were partitioned into four phases in

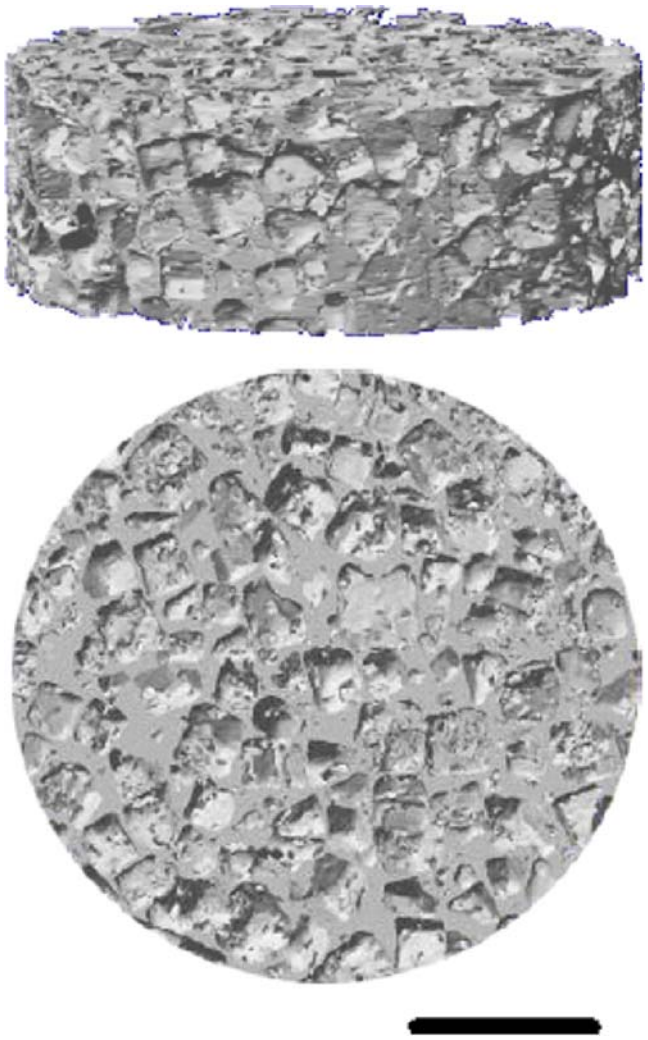

Fig. 2. Representative microCT scan of porous PPF scaffolds. Scaffolds were scanned at a 7 μm resolution with a voltage of 40 kV and current of 240 μA; thresholding was set from 55–255. Bar represents 2 mm.

accordance with previous investigations (15,18). A burst release at 24 h (Phase 1) was observed in all cases, and was generally followed by moderate or slow release. To describe this period, release rates for Phase 2 (days 1–3), Phase 3 (days 3–16), and Phase 4 (days 16–28) were calculated for all treatments (Table II).

As can be seen in Fig. 3, release from gelatin microparticles exhibited a burst release followed by slow to moderate release (<3% per day for Phase 2 and <1–2% per day for Phases 3 and 4) over the subsequent phases. We observed an overall effect of gelatin crosslinking, with increasing crosslinking extent from 10 mM to 40 mM glutaraldehyde resulting in decreased VEGF release (Table I, $p<0.05$). There was also a crosslinking effect on release rates within the four phases (Table II), although in the case of Phases 2 and 3, we noted increased release rates with higher crosslinking. However, for cumulative release at 28 days, lower crosslinking results in significantly higher release for equivalent doses (74.2±1.7% for 10 mM Low dose groups *vs.* 59.9±1.8% for 40 mM Low dose groups, $p<0.05$).

Growth factor dose also had a main effect on overall release as seen in Table I (higher doses show higher release, $p<0.05$), but for release rates, this was seen only in the early phases (burst release and Phase 2 release rates, Table II). Interestingly, burst release showed higher release with higher doses, while Phase 2 release rates showed the opposite effect with higher doses. Dose did not affect cumulative release at 28 days.

Finally, we saw a main effect of buffer type on VEGF release from the gelatin microparticles, with release profiles demonstrating higher release of growth factor in collagenase-containing PBS (Table I, $p<0.05$). However, for release rates, buffer type was significant only in the later phases (Phase 3

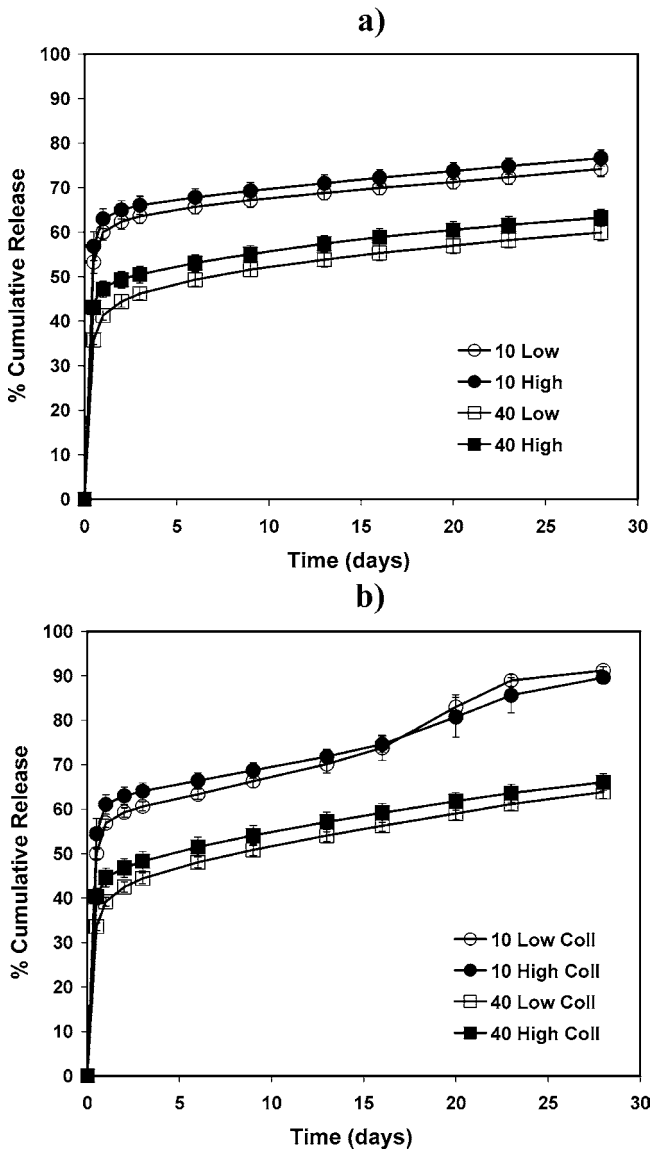

Fig. 3. *In vitro* VEGF release from gelatin microparticles. Average percent cumulative VEGF release from gelatin microparticles in **a** PBS buffer and **b** collagenase-containing PBS (Coll) as a function of gelatin crosslinking (10 mM *vs.* 40 mM) and growth factor dose (Low *vs.* High). Error bars represent means ± standard deviation for $n=6$.

Table I. Main Effects of Gelatin Crosslinking, Buffer Type, and Growth Factor Dose On Overall Release of VEGF

	Crosslinking	Buffer	Dose
Gelatin MPs	−	+	+
Composites—*In Vitro*	−	+	n/a
Composites—*In Vivo*	−	n/a	n/a

(+) and (−) denote increased and decreased effect as crosslinking was increased, collagenase was added to the buffer, or dose was increased ($p<0.05$); otherwise n/a if the parameter was not evaluated

and 10 mM groups in Phase 4) and on final cumulative release (74.2±1.7% in PBS *vs.* 91.2±0.9% in Coll for the 10 mM Low dose group).

In Vitro Release from Composite Scaffolds

In addition to microparticles, composite scaffolds of porous PPF with gelatin microparticles were tested *in vitro* (Fig. 4). In this case, only high doses of VEGF were used since growth factor dose only minimally affected initial release rates and not cumulative release at 28 days with the gelatin microparticles. As before, we saw the same effects of crosslinking and buffer type on overall release of VEGF over the entire release period as with the microparticles (Table I, $p<0.05$). For release rates, higher crosslinking in particular resulted in a significant effect on depressed burst release but increased Phase 4 release (Table II). For cumulative release at 28 days, higher crosslinking also resulted in decreased release (91.5±1.3% for 10 mM *vs.* 84.7±2.9% for 40 mM groups in PBS).

As for buffer effects (Table II), again we noted an increased effect on the last phase (specifically for 10 mM groups) and for cumulative release at 28 days (84.7±2.9% in PBS compared to 88.2±2.1% in Coll for 40 mM groups). However, instead of observing an effect in Phase 3 as with gelatin microparticles, it was Phase 2 release rates that were significantly different for buffer type. Additionally, it should be noted that although overall release profiles and release rates for Phase 3 and Phase 4 were similar when compared to gelatin microparticles alone, the cumulative release at 28 days was generally higher for the composite scaffolds.

In Vivo Release from Composite Scaffolds

Finally, composite scaffolds with 10 mM and 40 mM gelatin microparticles were also tested *in vivo* in a subcutaneous mouse model (Fig. 5). As with the previous release profiles, an overall effect of crosslinking on VEGF release was observed, with 40 mM crosslinking showing decreased growth factor release ($p<0.05$). While no significant differences of crosslinking were observed for burst release or Phase 2 rates, cumulative release was higher for 10 mM *vs.* 40 mM groups at day 14 (89.3±1.8% and 72.8±9.4%, respectively).

VEGF Bioactivity

A cell-based assay was used to evaluate the biological activity of VEGF released from the composite scaffolds. Controls included the treatment of HUVECs with media containing known amounts of VEGF while experimental groups received media collected after incubation with composite scaffolds. A linear, dose-dependent response of endothelial cell growth is evident from the controls (Fig. 6a). Growth factor bioactivity was evaluated as the ratio of observed cell growth for composite scaffolds to the expected cell growth (from the dose-dependence curve, calculated as the equivalent amount of soluble VEGF). The biological activity of VEGF was maintained above 90% of expected bioactivity at all times (Fig. 6b). VEGF released from both 10 and 40 mM composites between days 3–7 also showed enhanced bioactivity (over 150% of expected bioactivity) compared to days 1 and days 7–14 ($p<0.05$). There was no significant effect of microparticle crosslinking on VEGF bioactivity at each time point.

DISCUSSION

The objective of this study was to examine the effects of gelatin crosslinking, growth factor dose, and buffer type on VEGF release profiles from acidic gelatin microparticles

Table II. Release Rates of VEGF from Gelatin Microparticles and Composite Scaffolds

	Phase 1 (%/day)		Phase 2 (%/day)		Phase 3 (%/day)		Phase 4 (%/day)	
	PBS	Coll	PBS	Coll	PBS	Coll	PBS	Coll
Gelatin MPs	^,#	^,#	^,#	^,#	^,*			^
10 mM Low	60.0±1.8	56.9±0.9	1.8±0.1	1.9±0.1	0.5±0.0	1.0±0.1	0.4±0.1	1.5±0.2*
10 mM High	63.0±2.2	61.0±2.2	1.0±0.2	1.5±0.2	0.5±0.0	0.8±0.1	0.4±0.1	1.3±0.2*
40 mM Low	41.2±1.0	39.2±1.0	2.5±0.2	2.6±0.2	0.7±0.0	0.9±0.0	0.4±0.0	0.6±0.0
40 mM High	47.2±1.8	44.6±2.1	1.5±0.1	1.8±0.1	0.6±0.1	0.8±0.0	0.4±0.0	0.6±0.1
Composites—*In Vitro*	^	^	*				^	^
10 mM High	67.2±4.0	69.5±2.6	3.8±0.7	7.1±0.8	1.0±0.2	0.8±0.1	0.1±0.1	0.3±0.0*
40 mM High	59.4±4.4	59.8±6.3	3.6±0.9	5.8±1.7	1.0±0.3	0.9±0.2	0.4±0.1	0.4±0.0
Composites—*In Vivo*								
10 mM High	67.9±2.6		1.3±0.2					
40 mM High	64.3±4.9		0.8±0.7		n/a		n/a	

Average percent values (percent release per day) are given with standard deviations for an $n=6$ for microparticles and composites *in vitro*, and $n=3$ for composites *in vivo*. Statistical significance ($p<0.05$) between relevant groups for gelatin crosslinking (10 mM *vs.* 40 mM) is denoted by (^), for buffer type (PBS *vs.* Coll) by (*), and for growth factor dose (Low *vs.* High) by (#)

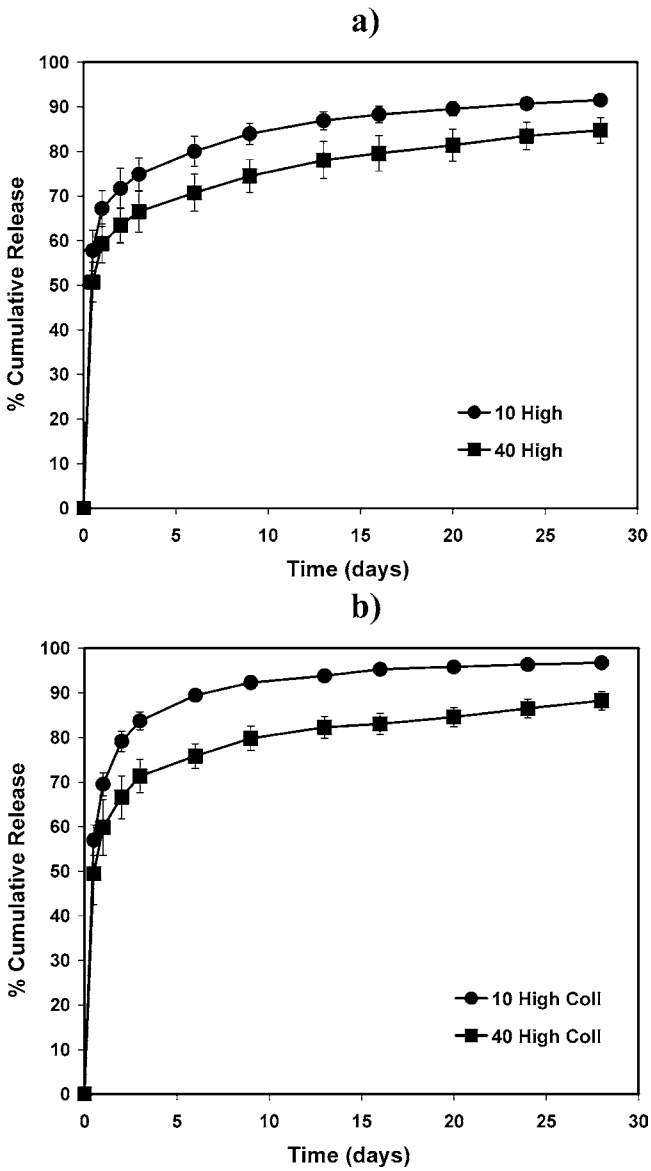

Fig. 4. *In vitro* VEGF release from composite scaffolds. Average percent cumulative VEGF release from composite scaffolds with gelatin microparticles loaded with a high dose of VEGF in **a**) PBS buffer and **b**) collagenase-containing PBS (Coll) as a function of gelatin crosslinking (10 mM *vs.* 40 mM). Error bars represent means ± standard deviation for $n=6$.

in vitro and *in vivo*. First, the acidic gelatin microparticles were synthesized in a water-in-oil emulsion and crosslinked with a low and high concentration of glutaraldehyde (10 and 40 mM). These microparticles were then assessed for the extent of crosslinking by swelling in PBS for 24 h. As previously demonstrated (18), the results show that more tightly crosslinked microparticles (40 mM) cannot absorb the same amount of water as lower crosslinked microparticles (10 mM) and therefore exhibited lower fold swelling ratios.

VEGF release from gelatin microparticles was then evaluated *in vitro* and a burst release was observed similar to what has been noted for VEGF released from gelatin disks (13). This burst was followed by slow to moderate release over 28 days, also similar to *in vitro* release profiles that have been examined with gelatin microparticles loaded with TGF-β1 in both PBS and collagenase buffers by Holland *et al.* (15). There was a significant effect of buffer type on growth factor release, illustrating that the addition of collagenase results in increased release of VEGF and confirming that gelatin undergoes enzymatic degradation as previously described (25). Also in the presence of collagenase, the crosslinking extent played a large role in determining release, suggesting that the less crosslinked 10 mM microparticles are more rapidly degraded while the more tightly crosslinked 40 mM microparticles can resist enzymatic degradation.

However, one difference between these studies and previous work by Holland *et al.* (15,18) was the significant effect of crosslinking observed not just in collagenase buffer but also in PBS alone, especially on burst release. This effect is not easily explained, since as described above, gelatin does not undergo hydrolytic degradation but rather enzymatic degradation, and the growth factor release is dependent largely on electrostatic interactions with the gelatin. However, the burst release largely reflects the equilibrium swelling of the microparticles, allowing for diffusion of any unbound growth factor out into the release medium. As noted earlier, higher crosslinking of the microparticles showed lower swelling ratios and thus a smaller burst release of VEGF. Additionally, tighter crosslinking of the gelatin produces smaller mesh size and can also affect the diffusion of the growth factor from the microparticles.

Finally, the differences between the two growth factors VEGF and TGF-β1 must also be considered. It is likely that VEGF associates differently than TGF-β1 does with acidic gelatin, and that this association can affect the equilibrium between bound and unbound growth factor. Previous work has shown that VEGF interaction with acidic gelatin is weaker than TGF-β1 (13); this may be due to differences in IEPs (8.6 *vs.* 9.5), sizes (38.2 kDa *vs.* 25 kDa), or tertiary structures (side-by-side *vs.* face-to-face beta sheets (26)).

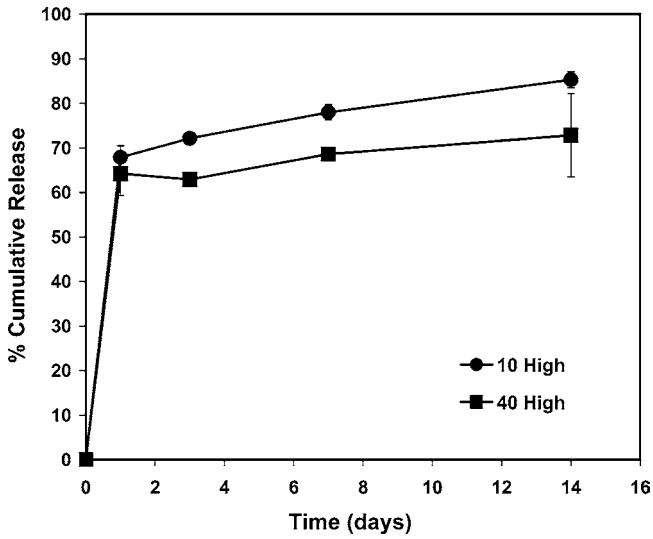

Fig. 5. *In vivo* VEGF release from composite scaffolds. Average percent cumulative VEGF release from composite scaffolds with gelatin microparticles loaded with a high dose of VEGF as a function of microparticle crosslinking (10 mM *vs.* 40 mM). Error bars represent means ± standard deviation for $n=3$.

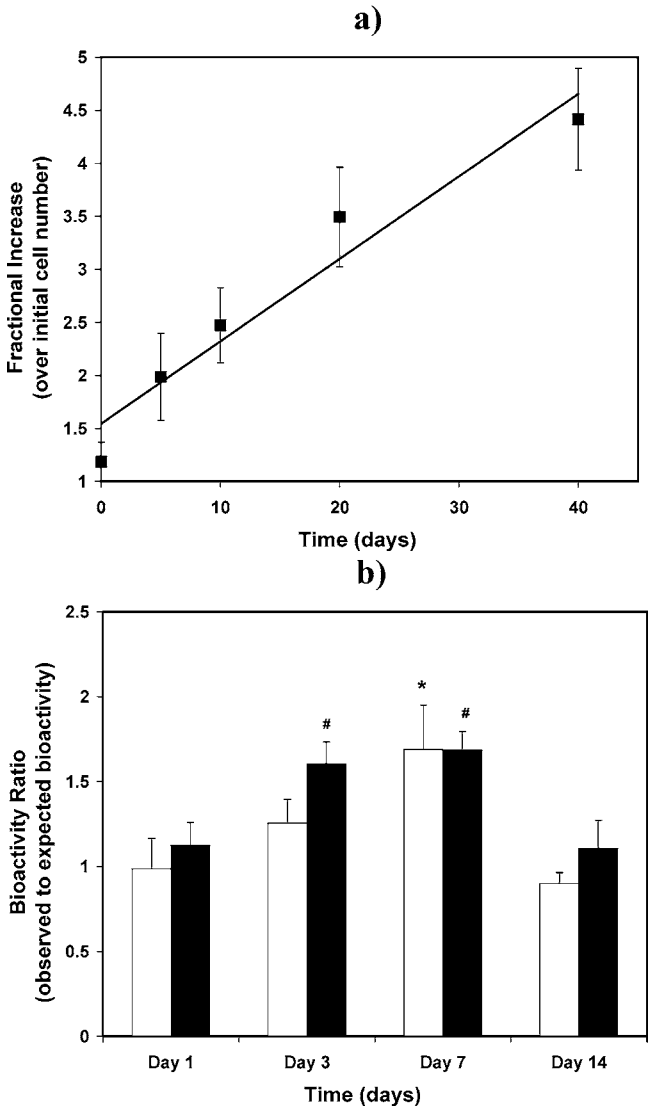

Fig. 6. VEGF bioactivity after release from composite scaffolds. **a**) A dose-dependent response on cell growth was observed for the controls receiving free VEGF and **b**) the fractional increase in cell growth with VEGF released from composites over controls for an $n=3$. Asterisks and sharp signs denote significant differences ($p<0.05$) for 10 mM (white bars) or 40 mM (black bars) groups, respectively, from their counterparts at both day 1 and day 14.

These differences between the two growth factors can account for the differences in their interactions with gelatin; for VEGF, other intermolecular forces such as hydrophobic and hydrogen-bonding interactions may be stronger than any electrostatic attractions between VEGF and gelatin. Thus, while the release kinetics of both growth factors from gelatin are similar, they are dependent on inherent properties of the specific growth factor and therefore not identical.

In addition to buffer type and gelatin crosslinking, we evaluated the effect of growth factor dose on VEGF release. The main effect was an increased release with a higher dose. However, the differences in burst release between groups of high and low doses were relatively small and not what one might have expected with a high dose being ten times larger than the low dose. Presumably, the ratio of free *vs.* bound growth factor is similar for both amounts and therefore, the effect on VEGF release is minimal for the investigated doses.

For *in vitro* release from composite scaffolds, we observed similar profiles to VEGF release from gelatin microparticles for different crosslinking extents in both PBS and collagenase-containing PBS. The main effects for gelatin crosslinking and buffer type were the same as with gelatin microparticles alone. We note that there were higher burst and cumulative releases from the composite scaffolds compared with the microparticles alone. Although the use of the Pluronic employed for the generation of the composite scaffolds and the presence of a porous polymer scaffold might have affected the overall release kinetics from the composites, no direct comparisons can be made between the two groups (composite scaffolds *vs.* microparticles) since the total amount of acidic and basic gelatin microparticles in the two groups was different.

In vivo release from composite scaffolds was also evaluated using a mouse subcutaneous model and we observed again the same main effect of crosslinking on overall release. However, there was no crosslinking effect on release rates for burst and Phase 2. Modeling an enzymatic environment with *in vitro* studies is at best a very rough prediction of what happens *in vivo*, and it may be that the protease environment at early times is different from what was modeled due to trauma and wound healing from surgery (27). We still note that increasing gelatin crosslinking resulted in significantly less growth factor release at 14 days, indicating that by varying crosslinking extent, this system can be used for the controlled delivery of VEGF *in vivo*. These results are similar to previous work that has utilized gelatin disks as a carrier for release of bFGF (28), or collagen hydrogels for VEGF release (7), which show that crosslinking extent can be varied to influence release of the growth factor *in vivo*.

Finally, the bioactivity of released VEGF was also assessed. For VEGF released from composite scaffolds, the bioactivity was maintained over 90% of expected bioactivity (equivalent amounts of soluble VEGF) at all time points. Additionally, we observed an enhancement in bioactivity at day 7 to over 150%. This suggests that the VEGF released from the composites is a combination of free VEGF and VEGF associated with gelatin fragments that act as a protective agent on the growth factor bioactivity, preserving and even enhancing its effect on endothelial cell proliferation. Similar effects on bioactivity have been reported when VEGF is released from alginate (10) and poly(lactide-co-glycolide) (29), but also from heparin composites (30), which is notable since the association of VEGF with heparin has been shown as a mechanism for maintaining its bioactivity *in vivo* (31).

CONCLUSIONS

This research demonstrates the efficacy of gelatin microparticles for achieving varied release profiles of VEGF over 4 weeks. The observed burst release in all cases is indicative of VEGF which is not associated with gelatin. The relative amount of growth factor associated with gelatin achieves an equilibrium value with no strong dependence on its dose. The release kinetics are characteristic of the specific growth factor due to the effects of growth factor size, charge, and conformation on its complexation with gelatin. The possible

association of released VEGF with gelatin fragments helps maintain its bioactivity. Finally, a systematic control of VEGF delivery can be achieved both *in vitro* and *in vivo* by altering the extent of microparticle crosslinking.

ACKNOWLEDGMENTS

The authors would like to acknowledge support of this work by a grant from the National Institutes of Health (R01-DE15164) (AGM) and by a National Science Foundation Graduate Research Fellowship (ZSP).

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