Fibrin-Loaded Porous Poly(Ethylene Glycol) Hydrogels as Scaffold Materials for Vascularized Tissue Formation

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Vascular network formation within biomaterial scaffolds is essential for the generation of properly functioning engineered tissues. In this study, a method is described for generating composite hydrogels in which porous poly(ethylene glycol) (PEG) hydrogels serve as scaffolds for mechanical and structural support, and fibrin is loaded within the pores to induce vascularized tissue formation. Porous PEG hydrogels were generated by a salt leaching technique with 100–150- μ m pore size and thrombin (Tb) preloaded within the scaffold. Fibrinogen (Fg) was loaded into pores with varying concentrations and polymerized into fibrin due to the presence of Tb, with loading efficiencies ranging from 79.9% to 82.4%. Fibrin was distributed throughout the entire porous hydrogels, lasted for greater than 20 days, and increased hydrogel mechanical stiffness. A rodent subcutaneous implant model was used to evaluate the influence of fibrin loading on *in vivo* response. At weeks 1, 2, and 3, all hydrogels had significant tissue invasion, but no difference in the depth of invasion was found with the Fg concentration. Hydrogels with fibrin loading induced more vascularization, with a significantly higher vascular density at 20 mg/mL (week 1) and 40 mg/mL (weeks 2 and 3) Fg concentration compared to hydrogels without fibrin. In conclusion, we have developed a composite hydrogel that supports rapid vascularized tissue ingrowth, and thus holds great potential for tissue engineering applications.

Introduction

BIO MATERIAL SCAFFOLDS ARE used in most tissue engineering approaches to provide mechanical and structural support for tissue ingrowth and development.¹ A three-dimensional biomaterial scaffold with high porosity and appropriate pore size is usually required for proper tissue reconstruction. The existence of interconnected pores allows extra space for tissue invasion and enhances mass transport, while the scaffold supports cell adhesion and migration.² Various natural and synthetic materials have been investigated for tissue engineering. Materials based on poly(ethylene glycol) (PEG) have received significant attention for a number of biomedical applications.³ PEG hydrogels with interconnected pores can be generated by particulate leaching, allowing good control of pore size based on the size of salt crystals used.⁴ Cell and tissue invasion has been observed within porous PEG hydrogels both *in vitro* and *in vivo*, with the invasion rate dependent on pore size.⁵

Regardless of the choice of biomaterial used to reconstruct tissues, stimulation of proper microvasculature formation is essential to success in tissue engineering. The formation of microvascular networks with perfusion is critical to the survival of new tissue.⁶ Current strategies for neovascularization have mostly focused on angiogenesis, which is the

formation of new capillaries by sprouting from existing microvasculature.⁷ Various genes or proteins of angiogenic factors, as well as cells involved in neovascularization, have been used to promote or accelerate vascularization in engineered tissues. However, limitations exist in those therapeutic strategies, including high cost and low stability in producing, handling, and storing the growth factors or cells involved. Moreover, the detailed mechanisms and potential adverse effects of delivering growth factors and cells remain largely unknown.⁸

An alternative approach to current angiogenic strategies is to use fibrin-based materials to induce neovascularization in the engineered tissue. Fibrin is a fibrous protein formed during wound hemostasis, in which the soluble blood plasma monomer fibrinogen (Fg) is polymerized into insoluble fibrin by thrombin (Tb).^{9,10} Fibrin and Fg play critical roles in blood clotting, cell–matrix interactions, inflammation, and wound healing.¹¹ Fibrin alone or in combination with other biomaterials has been used in multiple clinical and biomedical applications,^{12,13} including as a tissue sealant, for cell encapsulation,^{14,15} growth factor delivery,^{16,17} and as a tissue engineering scaffold.¹⁸ Fibrin gels also stimulate vascularization *in vitro*¹⁹ and *in vivo*.^{20,21}

In this study, a technique is described for generating composite hydrogels with porous PEG serving as the base

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scaffold and fibrin gels polymerized within the pores. The distribution of fibrin within the porous PEG hydrogels was evaluated using confocal microscopy and histology. Radiolabeling was used to assess loading efficiency and stability, and the influence of fibrin on the mechanical properties of the hydrogels was determined using compression testing. The effect of fibrin loading in porous PEG hydrogels on vascularization was investigated *in vivo*. These results show that fibrin can be loaded through porous PEG hydrogels and induce vascularization *in vivo*.

Materials and Methods

Materials

PEG (Mn 8000), Fg from human plasma (Fg, 50–70% protein), thrombin from human plasma (Tb), monoclonal anti- α -smooth muscle actin (SMA) produced in mouse (anti- α -SMA), 2-hydroxy-2-methylpropionophenone (Irgacure 1173), acryloyl chloride (98%), and triethylamine (TEA, 99.5%) were obtained from Sigma-Aldrich. Sodium chloride (99.5%), dichloromethane (99.9%), and ethyl ether (anhydrous) were obtained from Fisher Scientific. Alexa Fluor 647-conjugated Fg and Alexa Fluor 647-conjugated isolectin were obtained from Invitrogen. Goat anti-mouse immunoglobulin G (IgG); Fluorescein isothiocyanate (FITC) was obtained from AbD Serotec. Spectrozyme® TH was obtained from Sekisui Diagnostics, LLC.

Preparation of fibrin-loaded porous PEG hydrogels

A salt leaching technique that has been previously described^{4,5} was adapted to make porous PEG scaffolds containing Tb. Briefly, PEG-diacrylate (PEG-DA) was first synthesized by reaction of PEG (Mn 8000) with acryloyl chloride and TEA. The product was purified with dichloromethane dissolution and ethyl ether precipitation. Purified PEG-DA (250 mg/mL) was dissolved in saturated salt water, with Tb (100 U/mL) and Irgacure 1173 (0.5% w/v, photoinitiator) added to form the hydrogel precursor. Two hundred microliters of the precursor was mixed with 200 mg of sieved salt crystals (100–150 μ m in diameter) and polymerized under UV light (365 nm) for 5 min. The salt crystals were then leached out in 50 mL of deionized (DI) water with agitation for 3 h, with replacement of DI water every hour.

After salt leaching, excessive water on the hydrogels surface and inside pores was mostly removed by blotting with sterile gauze. The hydrogels were air-dried for 1 h to allow evaporation of water from the pores. Three hundred microliters of the Fg solution (10, 20, or 40 mg/mL) was dropped onto the porous hydrogel surface. The Fg solution was absorbed into the pores by gravity and capillary force. The hydrogels loaded with Fg were incubated at room temperature for 30 min to allow interaction of Fg with Tb to form the fibrin gel. The hydrogels were then rinsed with 5 mL of DI water to remove free Fg.

Visualization of fibrin in the pores

To observe the distribution of fibrin in the PEG scaffold, the Fg solution was initially mixed with 0.1 mg/mL Alexa Fluor 647 Fg conjugate before loading. The hydrogels were sectioned at different depths and imaged by confocal laser scanning microscopy with a 20 $\times$ objective, at 633-nm exci-

tation wavelength and with a 650-nm long-pass filter. Serial images were taken by a PASCAL laser scanning microscopy system and exported to Axiovision (Carl Zeiss AG) for three-dimensional reconstruction. The fibrin-loaded porous PEG hydrogels were also paraffin embedded, sectioned at 5- μ m thickness with microtome, and stained with hematoxylin and eosin (H&E). The sections were imaged by transmitted light microscopy with a 20 $\times$ objective (0.89 μ m/pixel).

Fg loading and release

Fg was radiolabeled with ¹²⁵I as described previously²² to quantify Fg within the hydrogels. ¹²⁵I-Fg (0.1 mg/mL) was mixed with the Fg solution (10, 20, or 40 mg/mL) and loaded into porous PEG hydrogels as described above. Pure fibrin gels (300 μ L each) with 10, 20, or 40 mg/mL Fg and 100 U/mL Tb were used as controls. Labeled Fg within each individual hydrogel was quantified with a γ counter before and after washing with phosphate-buffered saline (PBS). Fibrin loading efficiency (*E*) was calculated as

$$E = \frac{F_{\text{after}}}{F_{\text{before}}} \times 100,$$

where *E* is the fibrin loading efficiency, and *F*_{before} and *F*_{after} are the radioactivity of each hydrogel before and after washing, respectively. Each value was averaged from three parallel studies (*n* = 3).

To test the bioactivity of Tb within porous PEG hydrogels, Spectrozyme TH was diluted to 1.5 mM with PBS and mixed with equal volume of Tb serial dilutions (1–100 U/mL) or porous PEG hydrogels (with 100 U/mL Tb in precursor, *n* = 3). After 30 min of incubation, the solutions were read at 405 nm with spectrophotometer (Molecular Devices). The bioactivity of Tb within porous PEG hydrogels was calculated by comparing with the standard curve obtained from Tb serial dilution.

The release of Fg from fibrin gels was measured during incubation. Briefly, the fibrin-loaded porous PEG hydrogels and pure fibrin gel controls (*n* = 3) were incubated in 5 mL PBS (pH 7.4) at 37°C. The amount of protein released was measured with a γ counter as described above. Neither enzymes nor enzymatic inhibitors were added to the PBS in this study.

Mechanical test

The fibrin-loaded hydrogels with 10, 20, or 40 mg/mL Fg were made into a cylindrical shape (~10 mm in diameter, ~7 mm in height) and compressed by an Instron 4465 compression tester (Instron Corp.). Porous PEG hydrogel without fibrin was used as a control. A stress(σ)–strain(ϵ) curve was plotted for each hydrogel. There is a linear region of each curve, where the material follows Hooke's Law ($\sigma = E\epsilon$), where σ is stress ($\frac{F}{A}$) and ϵ is strain ($\frac{L-L_0}{L_0}$). For consistency, we chose to use the data obtained at strain ranges from 0 to 0.1, and calculated the slope of this region using a linear regression to determine the Young's modulus (*E*). Each value was averaged from five parallel studies (*n* = 5).

Animal model

All animal experiments were carried out at Edwards Hines, Jr. VA Hospital and all procedures approved by the Institutional Animal Care and Use Committee. Fibrin-loaded

PEG hydrogels with 0, 10, 20, and 40 mg/mL Fg, and a fibrin gel control (20 mg/mL Fg with 100 U/mL Tb) ($n=5$) were synthesized and prepared under sterile conditions. Polypropylene shells were fabricated in the shape of a top hat, with a height of 4 mm, a diameter of 10.5 mm, and an integral sewing ring extending from the open end of the hat. The shells were autoclaved for sterilization before animal surgery and all hydrogels were made to fit in the shells.

A rodent subcutaneous implantation model was used for evaluating our materials *in vivo*.²⁰ The model allows unidirectional tissue ingrowth with the biomaterial with minimum background inflammation. Briefly, male Lewis rats (300–400 g, $n=5$; Charles River) were anesthetized initially with 5% isoflurane via a nose cone and maintained at 2–4% isoflurane/35% oxygen mixture during the procedure. Their backs were shaved and skin scrubbed with isopropyl alcohol followed by a povidone-iodine antiseptic solution. A longitudinal incision was made along the spine and the skin separated using blunt dissection. Polypropylene shells containing the hydrogels were implanted subcutaneously and secured with four evenly spaced sutures through the sewing ring. Each rat received six implants with the implant location determined randomly. The skin incision was closed using a 4-0 nylon suture.

At 1, 2, and 3 weeks after the implantation, Alexa Fluor 647-conjugated isolectin was injected to the animals via the tail vein under general anesthesia. The animals were then perfusion fixed with 4% formaldehyde and the implanted samples harvested. The harvested tissue, including the entire hydrogel and underlying muscle, were cut into two symmetric pieces. One half was paraffin embedded and sectioned (5- μ m thickness) for histological staining. The other half was embedded in Optimal Cutting Temperature compound, frozen, and sectioned (50- μ m thickness) for immunofluorescent staining and imaging. Sections were cut through the center of the sample so they contained cross sections of the gel with the interface of the underlying muscle. The examiners were blinded during all animal surgeries as well as image analysis.

Histological analysis

Paraffin-embedded tissue sections were stained with H&E and Masson's trichrome. For quantification of tissue invasion,

the H&E stained sections were digitally imaged (5 $\times$ objective, 1.26 μ m/pixel) using an Axiovert 200 inverted microscope. The depth of tissue invasion was defined as the maximum depth of tissue ingrowth within the pores from the underlying muscle. Masson's trichrome stained sections were used to visualize fibrin remaining within the pores and collagen deposition.

Vascular analysis

To visualize the microvasculature, Alexa Fluor 647 isolectin (far red) was perfused before harvesting the samples. The lectin is selectively taken up by endothelial cells. Frozen sections were then stained with FITC for SMA using an indirect immunostaining procedure. Briefly, tissue sections were first incubated with a primary antibody (anti- α -SMA produced in mouse, 2 μ g/mL) and 10% goat serum at 4°C. After overnight incubation and rinsing with PBS, a secondary antibody (goat anti-mouse IgG: FITC, 2.5 μ g/mL) was then added and incubated for 2 h at room temperature. The hydrogel sections were imaged near the tissue interface with confocal microscopy (Carl Zeiss AG) with dual fluorescence simultaneously for both endothelial cells (633-nm excitation, 650-nm long-pass filter, far red) and smooth muscle cells (SMCs) (488-nm excitation, 505–530-nm band-pass filter, green) with a 20 $\times$ objective. The images were analyzed for vascular density based on the following equations:

$$\text{Vascular Density} = \frac{\text{Area of lectin positive staining}}{\text{Area of tissue}}$$

Statistical analysis

All statistical data are expressed as means $\pm$ standard deviation. Data were analyzed by a one-way analysis of variance with a Tukey-Kramer post test using SigmaStat. Values of $p < 0.05$ were considered statistically significant.

Results

Visualization of fibrin in pores

PEG hydrogels with 100–150- μ m pore size were chosen for the hydrogel conditions due to previous results in which these hydrogels were shown to support vascularized tissue

FIG. 1. Fibrinogen (Fg) is distributed throughout the volume of porous PEG hydrogels. **(A)** 3D volume rendering of fluorescence Fg in porous PEG hydrogels imaged using confocal microscopy. **(B)** An H&E stain of fibrin (pink)-loaded porous PEG (purple) hydrogels. Fg was observed throughout the entire thickness of hydrogel cross sections. The hydrogels were 10 mm in diameter and 5 mm in height. Scale bar = 100 μ m. Fg concentration = 40 mg/mL. PEG, poly(ethylene glycol); H&E, hematoxylin and eosin. Color images available online at www.liebertpub.com/tea

formation in a skeletal muscle wound model.⁵ Tb (100 U/mL) was preloaded in the scaffold to catalyze the polymerization of Fg to fibrin. Fibrin was visible in pores throughout the entire volume of the hydrogel (Fig. 1). Fibrin was observed in pores within the hydrogel by reconstruction of serial images of fluorescently labeled Fg by confocal microscopy (Fig. 1A). Histological staining of fibrin/PEG composites further confirmed the distribution of fibrin within porous PEG hydrogels, (Fig. 1B). With both staining methods, fibrin was visualized throughout the porous hydrogels, with no visible difference in spatial distribution.

Fibrin loading efficiency

Fg (10, 20, and 40 mg/mL) was mixed with ¹²⁵I-Fg conjugate (0.1 mg/mL) before polymerization to quantify incorporation efficiency. No significant difference in encapsulation efficiency in porous PEG hydrogels was found between Fg concentrations with a range of 79.9–82.4% (Table 1). There was a greater incorporation of Fg within pure (noncomposite) fibrin gels with efficiency ranging from 98.7% to 99.0%. This presumably results from reduced availability of Tb when incorporated in the PEG scaffold compared to the same concentration of Tb in a solution. Tb within porous PEG hydrogels exhibited a reduced activity ($8.22 \pm 2.38\%$) relative to a free Tb solution (100 U/mL).

Fibrin elution in vitro

The hydrogels were incubated in PBS (pH 7.4) under infinite sink condition at 37°C in the absence of enzymes or enzyme inhibitors. ¹²⁵I in the surrounding buffer was quantified for both fibrin/PEG composites and pure fibrin gels to determine the amount retaining in the gels (Fig. 2). Due to the lower encapsulation of Fg, the initial amount was lower in composite hydrogels than pure fibrin gels at all concentrations. However, there was a rapid decrease in Fg levels in pure fibrin gels in the first 2 days. Fg levels in composite hydrogels were significantly greater than pure fibrin gels with 40 mg/mL (Fig. 2A), 20 mg/mL (Fig. 2B) and 10 mg/mL (Fig. 2C) initial Fg loading. For fibrin gels, Fg levels decreased rapidly within the first week, with less than 5 mg/mL Fg remaining after day 7 and nearly completed degradation by 20 days. For all composite hydrogels, 5 mg/mL of Fg remained after 20 days. Therefore, Fg was retained within composites hydrogels longer than in pure fibrin gels.

Mechanical properties of fibrin/PEG composites hydrogel

Compression testing was performed on the hydrogels to determine the influence of fibrin loading within the

FIG. 2. Fg remaining within composite hydrogels and pure fibrin generated using (A) 10 mg/mL, (B) 20 mg/mL, and (C) 40 mg/mL initial Fg concentration. The samples were incubated at 37°C in saline in the absence of enzymes or inhibitors. *Indicates significant difference in Fg remaining ($p < 0.05$, $n = 3$).

pores on composite hydrogel properties. The presence of fibrin in the pores increased stiffness relative to control porous gels (Fig. 3). In addition, the compressive modulus of the hydrogels increased with the Fg concentration.

TABLE 1. FIBRINOGEN ENCAPSULATION EFFICIENCY

Fg concentration (mg/mL)	Composite hydrogels	Pure fibrin gels
10	$81.3 \pm 13.4\%^a$	$98.9 \pm 0.1\%$
20	$82.4 \pm 7.1\%^a$	$98.7 \pm 0.7\%$
40	$79.9 \pm 2.6\%^a$	$99.0 \pm 0.4\%$

^aIndicates difference ($p < 0.05$) between composite hydrogels and pure fibrin gels at the same concentration.

FIG. 3. Compressive moduli of fibrin-loaded porous PEG hydrogels with varying Fg concentration show that the presence of fibrin gels increases stiffness in a dose-dependent fashion. *Indicates $p < 0.05$ ($n = 5$).

Histological analysis in vivo

Porous PEG hydrogels with varied Fg loading concentrations (0, 10, 20, and 40 mg/mL) were implanted subcutaneously in rats, while pure fibrin gels (20 mg/mL Fg + 100 U/mL Tb) served as a positive control. The animal model was selected for its easy use, minimal background inflammation, and unidirectional tissue interaction with the biomaterial due to the polypropylene shell (Fig. 4). At weeks 1, 2, and 3, all implanted samples were harvested and processed for histological analysis. Masson's Trichrome and H&E staining was performed for evidence of tissue invasion, inflammation, and quantification of tissue invasion depth (Fig. 5). For porous PEG hydrogels with and without fibrin loading, tissue invasion was observed in the pores of all porous hydrogels (Fig. 5A). At regions near the hydrogel and muscle interface, tissue invaded the pores and the fibrin was remodeled into collagen-rich vascularized tissue (Fig. 5C). Interestingly, fi-

brin was still present in the pores at regions far from the muscle interface (Fig. 5D). For the pure fibrin gel implants, tissue did not invade into the bulk gel. Instead, a layer of inflammatory tissue was formed at the tissue-fibrin interface (Fig. 5B) as the fibrin gels degraded and tissue formed at the muscle surface.

Tissue development and collagen production can be observed within the pores of the PEG scaffolds (Fig. 6). At all time points, a layer of inflammatory tissue (red) was present at the tissue-polymer interface, while granulation tissue (blue) was present in the center of the pores. The collagen density within the pores increased from 1 (Fig. 6A) to 2 (Fig. 6B) and 3 weeks (Fig. 6C). The development of tissue within the pores progressed from characteristics of acute inflammation (week 1) to granulation tissue rich in collagen that appeared histologically similar to tissue formed in response to pure fibrin gels.

The depth of tissue invasion from the underlying skeletal muscle into fibrin-loaded porous hydrogels was quantified (Fig. 7). Only samples from porous PEG hydrogels were evaluated, as tissue did not invade into the bulk structure of fibrin gels. Tissue invasion was observed within the pores of all hydrogels at weeks 1, 2, and 3. No significant differences in the depth of tissue invasion were found among groups with the Fg concentration at any time (Fig. 7A). However, when comparing the same group at different time points, the group without fibrin (0 mg/mL) did not vary over time. For the groups with fibrin (10, 20, and 40 mg/mL), there was a steady increase in the depth of invasion over time (Fig. 7B).

Vascular analysis in vivo

All animals were injected with fluorescently labeled isolectin (Alexa Fluor 647) and perfusion fixed with 4% formaldehyde before tissue harvest to evaluate vascularization within the pores. Vessels were observed for all regenerated tissue induced by composite hydrogels or pure fibrin gels (Fig. 8). For porous PEG hydrogels without fibrin (– control; Fig. 8A, D, G), there was vessel formation, but the density

FIG. 4. Images of implants in the rodent subcutaneous implantation model at 1 week postimplantation. (A) The polypropylene shell was implanted against the underlying muscle. (B) The hydrogel can be seen adhering to the underlying muscle following removal of the shell. Color images available online at www.liebertpub.com/tea

FIG. 5. Masson's trichrome staining of implanted (A, C, D) composite and (B) pure fibrin imaged using 5× (A, B) and 20× (C, D) objectives. For composite hydrogel implant (A), host skeletal muscle can be seen underlying the implants, and vascularized tissue invasion can be seen within pores near the interface. Far from the interface, fibrin still resides within the porous gels, where tissue has not invaded. For pure fibrin gel implant (B), a thick layer of inflammatory tissue formed on top of muscle tissue. High-resolution images allow detail of the vascularized tissue with high collagen deposition within the pores near the muscle (C) and residual fibrin deep within the pores (D). Scale bars = 200 μm. Color images available online at www.liebertpub.com/tea

appeared to be low, while for fibrin-loaded porous PEG hydrogels (Fig. 8B, E, H) more vessels can be seen inside pores. Pure fibrin gels (+ control; Fig. 8C, F, I) induced a high density of vessels formation at the interface. All tissue samples were simultaneously stained for α -SMA. In the under-

lying skeletal muscle, large diameter vessels were surrounded by vascular SMCs (Fig. 9A). However, in the newly generated tissues induced by fibrin gels (Fig. 9B) and composite hydrogel (Fig. 9C), minimal interactions between vessels and SMCs were observed.

FIG. 6. Masson's trichrome staining of implanted fibrin/PEG composites (A–C) and pure fibrin gels (D–F) at 1 (A, D), 2 (B, E), and 3 (C, F) weeks after implantation. Inflammatory tissue (red) presents at interface in pores and becomes less over time, while collagen stain (blue) becomes thicker and darker as collagen fibers mature over time. Scale bar = 50 μm. Color images available online at www.liebertpub.com/tea

FIG. 7. Depth of tissue invasion of within porous PEG hydrogels plotted with time at different Fg concentration. There were no significant differences in depth of invasion with Fg concentration (**A**), but invasion depth increased with time for Fg-loaded hydrogels (**B**). *Indicates $p < 0.05$ ($n = 5$).

Vascular density was quantified based on the dual fluorescent images (Fig. 10). For fibrin-loaded porous PEG hydrogels, vascular density was quantified as newly generated tissues in pores near the tissue/hydrogel interface. A significantly higher vascular density could be found with fibrin incorporation in porous PEG hydrogels at week 1 (20 mg/mL), week 2 (40 mg/mL), and week 3 (40 mg/mL) relative to control hydrogels without fibrin. Fibrin gels alone had a greater vascular density than empty hydrogels at all time points. While not significantly different, fibrin gels induced a greater mean vessel density than fibrin-loaded hydrogels at 1 week (pure fibrin $8.93 \pm 5.33\%$ vs. fibrin/PEG $5.43 \pm 0.92\%$, $p = 0.19$), but by 3 weeks the density levels were similar

between the groups (pure fibrin $8.15 \pm 1.92\%$ vs. fibrin/PEG $6.17 \pm 0.94\%$, $p = 0.08$).

Discussion

In this research, we describe a method for the generation of composite hydrogels consisting of a natural material with angiogenic properties (fibrin) loaded in the pores of a synthetic polymer with controllable chemical and mechanical properties (PEG). The hydrogels are easy to form, are biocompatible, and stimulate vascularized tissue formation upon implantation. We have previously shown that porous PEG hydrogels can permit the formation of vascularized

FIG. 8. Vascularized tissue formation within porous PEG hydrogels. Confocal microscopy images of isolectin (red)-labeled blood vessels in porous PEG without fibrin (**A, D, G**), porous PEG with 20 mg/mL (**B**), and 40 mg/mL (**E, H**) Fg, and pure fibrin gel (**C, F, I**) at weeks 1 (**A–C**), 2 (**D–F**), and 3 (**G–I**). Green color is the autofluorescence of native tissue. Scale bar = 100 μ m. Color images available online at www.liebertpub.com/tea

tissues.⁵ However, PEG hydrogels are inert and require additional signals to stimulate a significant biological response. The presence of fibrin in the pores resulted in greater vascularization than PEG gels alone (Fig. 10).

Fibrin gels alone have been explored as scaffolds for tissue engineering applications. However, their rapid degradation typically leads to a loss of tissue volume limiting the size of defects that can be treated. In addition, it is difficult to precisely control the mechanical properties of fibrin gels or maintain the scaffold volume during tissue ingrowth. The

PEG gel provides controllable mechanical properties and allow for maintenance of the sample volume, while vascularized tissue invades. Compared to fibrin gel alone, the porous PEG hydrogel hindered Fg release, extending the presence of fibrin (Fig. 2). This composite system prolongs the availability of the Fg signals and allows for vascularized tissue ingrowth, while maintaining bulk tissue volume. Future studies will involve PEG-based copolymer systems, in which the influence of controlled degradation on the stability of the vascularized tissue are investigated.

FIG. 9. Immunofluorescence staining of α -SMA (fluorescein isothiocyanate, green) of isolectin (Alexa Fluor 647, Red) perfused tissue samples in native muscle tissue (**A**), and regenerated tissue induced by pure fibrin gel (**B**) and composite hydrogel (**C**). Arrows indicate positive staining for α -SMA lining a large vessel. Scale bar = 100 μ m. α -SMA, smooth muscle actin. Color images available online at www.liebertpub.com/tea

FIG. 10. Vascular density was increased due to the presence of fibrin in the pores. The plot shows density within porous hydrogels versus time at different Fg loading concentrations. *Indicates $p < 0.05$ ($n = 5$).

Synthetic polymer systems other than PEG could be investigated as the bulk porous polymer. However, PEG-based hydrogels have been investigated for a number of tissue engineering applications due to their biocompatibility and their tunable mechanical and physical properties. In comparison to other composite systems, such as fibrin-loaded porous poly glycolic acid (PGA) or poly-L-lactic acid (PLLA) scaffolds,^{14,18} a significant advantage of our system is that the fibrin is easily loaded throughout the entire porous volume of the PEG scaffold. Due to the hydrophilic nature of PEG, Tb could be preloaded in the precursor solution for loading throughout the scaffold. When the Fg solution was added, it was rapidly absorbed into the scaffold and polymerized in the pores, resulting in extensive loading. When a hydrophobic scaffold, such as PLLA is used, the distribution of fibrin throughout the entire porous scaffold is challenging. Tb activity is lost due to the solvents used for scaffold fabrication, resulting in the requirement that Tb is mixed with Fg immediately before loading. This limits the application to low concentrations of Tb and Fb to avoid rapid gelation during fibrin loading. The rapid polymerization of fibrin relative to the adsorption rate in these hydrophobic scaffolds may also lead to incomplete loading. Finally, in this study, we were interested in the study of vascularization of a porous scaffold loaded with fibrin in the absence of degradation. This is not easy to accomplish with PLLA or PGA, which degrade via hydrolysis. PEG hydrogels, however, do not degrade *in vivo*, which allows for the study of the influence of fibrin in the absence of bulk polymer degradation.

Comparing fibrin loaded in porous PEG hydrogel with pure fibrin gels, there was ~20% Fg that was not polymerized in the hydrogel (Table 1). This could be due to the lack of availability of Tb in hydrogel network compared to the direct mixture, as well as the potential deactivation of Tb caused by a high salt concentration and UV exposure. The degradation of fibrin *in vivo* is caused by plasmin-catalyzed lysis, or fibrinolysis.⁹ However, in our *in vitro* release study, no enzyme or enzyme inhibitor was added in the system. So the release was more likely from dissolution of fibrin, rather than from degradation of fibrin into soluble fibrin fragments, caused by enzymes. This result agrees with previous results,

where little difference was found in fibrin gel degradation in the absence or presence of an enzyme inhibitor.²³ Interestingly, the presence of fibrin at times greater than 3 weeks (21 days) was also observed *in vivo*. The regions far from the interface still contained fibrin as they had not been exposed to proteolytic enzymes. This could be explained by the hindered diffusion of the degraded fibrin from the porous structure. Regardless, the prolonged presence of fibrin could allow for long-term delivery of factors and cells that promote greater vascularized tissue ingrowth and development.

A number of studies have evaluated the mechanical properties of fibrin gels, with Young's moduli ranging from 10^4 to 10^6 Pa, depending on the concentrations of Tb and Fg, as well as polymerization conditions, such as pH, ionic strength.^{24–26} Both ultimate tensile strength and Young's modulus increase with Fg and Tb concentrations.²⁷ A similar effect was observed in our composite hydrogels, in which Young's modulus increased with the Fg concentration (Fig. 3). This provided further evidence that Fg polymerized into fibrin upon loading, increasing the mechanical properties of the bulk polymer gels.

For *in vivo* evaluation, a rodent subcutaneous implantation model was used to evaluate vascularized tissue ingrowth. Compared with a previous muscle wound model in which porous hydrogels were inserted inside a pocket between the fascia and muscle,⁵ the current model causes minimal trauma resulting in a very low background inflammatory response. In the previous model, blood and body fluid could easily fill in the pores during the surgical implantation, which was believed to have contributed to the high levels of tissue ingrowth observed in that system. With the animal model used here, a limited amount of blood was present on the surface of muscle tissue during implantation, which largely reduced the high baseline that can occur in wound-healing or highly inflammatory environments. This allows for an extensive evaluation of the inductive effects of the loaded scaffolds that is useful for large animal preclinical models of engineered tissue formation, where the baseline tissue growth is low.²⁸

Surprisingly, the porous PEG hydrogels without fibrin loading still had levels of tissue invasion similar to fibrin-loaded gels (Fig. 7). This may be due to the presence of Tb within the gels. Porous PEG hydrogel implantation without Tb exhibited very little invasion at the hydrogel/tissue interface at weeks 1, 2, and 3 (data not shown). The presence of Tb, even in the absence of Fg, could affect the biological activity of the material and cause more tissue interaction, due to the active roles of Tb in multiple biological processes.^{29,30} The incorporation of fibrin in pores affected the mechanical properties, and possibly transport within, the hydrogels, which could initially restrict tissue invasion. Even though the presence of Fg did not increase the depth of tissue invasion, the vascular density was increased significantly due to fibrin. The highest vascular density was found at the newly generated tissues induced by pure fibrin gel (+control), while the lowest was at porous PEG hydrogels without fibrin loading (Figs. 8 and 10). A higher density of perfused vascular network provides better mass transport for oxygen, nutrients, and cells, as well as more efficient removal of metabolic wastes, and therefore, could potentially allow further tissue invasion given longer time.

Several limitations still exist in our current study, and therefore require further investigation. First, while fibrin

clearly increased vascularized tissue formation, it is not clear whether or not the tissue would be stimulated to continue to invade and vascularize the entire porous hydrogel. The depth of invasion can be increased by incorporating growth factors that induce tissue growth and vessel formation, such as fibroblast growth factor family and vascular endothelial growth factor. Fibrin has been shown to be a good delivery system for growth factors¹⁷ and its sustained presence in the gels could be used to deliver factors that promote greater ingrowth. Second, the stability of the new vessels also needs further improvement. Immunofluorescent staining for α -SMA showed only limited SMCs coverage for the new vasculature (Fig. 9). Lack of SMC association with new vessels suggested of the absence, or delay, of the vessel maturation process.⁸ Growth factors that favor vascular stabilization, such as platelet-derived growth factor family, angiopoietin-1, and transforming growth factor- β , could be delivered to induce vessel maturation and stabilization. Last, the porous PEG scaffold is stable and does not degrade in physiological conditions. The introduction of chains degradable by hydrolysis or enzymatic degradation should be introduced into the material, so that it could eventually all be replaced with native tissue. The rate of degradation, however, needs to be carefully controlled to allow sufficient tissue regeneration before scaffold degradation.

Conclusion

In this study, a technique for generating composite materials of porous PEG hydrogels loaded with fibrin gels is described. The effect of fibrin loading was evaluated *in vitro* and *in vivo*. The presence of fibrin within the pores increased hydrogel stiffness, while the presence of porous PEG delayed fibrin breakdown. The materials supported tissue invasion and increased vascular network formation *in vivo*.

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Disclosure Statement

No competing financial interests exist.

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