Published in final edited form as:

J Biomed Mater Res A. 2010 October ; 95(1): 283–293. doi:10.1002/jbm.a.32825.

A novel composite construct increases the vascularization potential of PEG hydrogels through the incorporation of large fibrin ribbons

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Abstract

Developing a mechanism to vascularize tissue engineered constructs is imperative for transplant function and integration, particularly when delivering hypoxia-sensitive tissues such as pancreatic islets. Previous efforts have focused on bulk modifications of scaffold materials rendering the entire construct permissive to vessel penetration or the formation of a porous structure where vessels can infiltrate the empty spaces. Here we describe a novel construct composed of large fibrin ribbons encapsulated within a poly(ethylene glycol) (PEG) hydrogel. The PEG/fibrin ribbon composite scaffold facilitates co-culture of adhesive and non-adhesive cell types, thus providing closely neighboring environments with distinct material properties specific to the needs of two clinically relevant cell populations. This advantage is demonstrated here by the successful co-culture of pancreatic islets in the PEG component and vessel-forming endothelial cells in entrapped fibrin ribbons. Transplanted endothelial cells can form anastomoses with host vasculature, suggesting that our co-cultures may lead to more rapid scaffold vascularization. Additionally, we show that surface-seeded endothelial cells form multicellular projections that migrate into non-adhesive PEG hydrogels along permissive fibrin ribbons, further demonstrating composite construct vascularization potential. Distribution of large fibrin ribbons throughout PEG hydrogels provide a potential mechanism for vascularization of a well-established biomaterial without inherently changing its desirable properties.

Keywords

PEG hydrogel; fibrin ribbons; scaffold vascularization; co-culture; endothelial cells

Introduction

Tissue engineered materials have the potential to restore function to or replace diseased organs such as the pancreas, heart, skin, and cartilage¹. Poly(ethylene glycol) (PEG) hydrogels are one commonly-used material for tissue engineering applications as they offer a mechanically-stable, biocompatible three-dimensional platform in which to encapsulate and culture *in vitro*, or implant *in vivo*, a target cell population^{2–6}. PEG hydrogels are also highly resistant to protein and cell adhesion, and thus provide an immunoprotective barrier between immune cells and encapsulated cells⁷. Although beneficial in many respects, the non-adhesive nature of PEG hydrogels renders them incapable of construct vascularization without significant modifications. Vascularization of engineered constructs is important for

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viability and integration of transplanted cells⁸. Among necessary nutrients for cell growth, oxygen is typically limiting due to its low tissue solubility⁹, requiring <200µm between target cell types and a blood vessel⁸. To protect metabolically active tissues from hypoxia-induced necrosis, oxygen diffusion limitations necessitate often impractically thin constructs or modifications to allow for vessel penetration. Providing a mechanism for rapid vascularization of implanted constructs is of particular importance to highly hypoxic sensitive tissues such as pancreatic islets¹⁰.

Several mechanisms have been employed to enhance the vascularization potential of synthetic biomaterials, including incorporating large pores into the scaffold to facilitate vessel in growth and modifying hydrogels with endothelial cell adhesive substrates that render the hydrogel construct permissive to vessel penetration^{8,11–13}. Both of these approaches achieved considerable success in encouraging capillary formation from cultured endothelial cells *in vitro* and promoting significant angiogenesis *in vivo*; however, limitations in these scaffolds remain. For example, cytotoxic steps are required to generate macroporous constructs, precluding the encapsulation of cells within the bulk matrix. This design relies on cell seeding into the pores which is not conducive to the culture or implantation of self-associating and relatively non-adherent tissues such as pancreatic islets. Additionally, seeded cells are unprotected from the host immune system when implanted *in vivo*. In contrast to porous scaffolds, covalent attachment of fibrinogen molecules to photopolymerizable PEG macromers enables both cytocompatible cell encapsulation and endothelial cell penetration via endothelial cell attachment sites¹³. One potential limitation of this system, however, is the resulting construct homogeneity. Encapsulated target cells may require a chemical environment unique from that which would be most conducive for angiogenesis. Much research has sought to identify material properties appropriate to maintain viability and function of specific cell populations for tissue engineering applications. Thus, the design of a composite construct supporting vascularization while providing a distinct but closely neighboring environment for the target tissue/cells would be beneficial. Such a construct would bring blood vessels within a close enough proximity to allow for sufficient oxygen and nutrient diffusion to transplanted cells, but also could provide a unique supportive environment to optimize target cell viability and function.

A second advantage to a composite construct with two distinct, but closely associated and interweaving, compartments would be the opportunity to co-culture multiple cell populations, including adherent and non-adherent dependent cell types. In the case of scaffold vascularization, a composite construct could be utilized to co-culture functional target tissue for disease amelioration in one scaffold component and vessel-forming endothelial cells in the other. Transplanted endothelial cells and pre-formed capillary-like structures are capable of anastomosing with host vessels leading to enhanced implant vascularization^{14,15}. Therefore it would be attractive to co-culture target cells and endothelial cells *in vitro* prior to implantation of the construct to enable endothelial cell proliferation and subsequent formation of interconnected, vessel-like structures. This pre-implantation endothelial cell development would potentially facilitate rapid construct vascularization and diminish the duration of target cell hypoxia *in vivo*.

In this study we have developed a composite hydrogel culture platform consisting of large fibrin ribbons dispersed and subsequently encapsulated within a PEG hydrogel. This composite construct allows for the co-culture of two distinct cell types, specifically adherent- and non-adherent-dependent populations. Several experiments were performed to highlight the utility of these culture platforms for both potential vascularization of PEG hydrogels and co-culture applications. First, fibrin ribbon size distribution was characterized to verify that fibrils could be generated that were large enough to support both vascularization and/or an encapsulated cell population. Second, we demonstrated that

adherent-dependent fibroblasts and endothelial cells could be encapsulated and cultured within the fibrin ribbons distributed in the PEG matrix. Third, we explored vascularization potential by seeding endothelial cells onto the surface of the composite constructs and characterizing subsequent cell penetration. Finally, we demonstrated that two distinct cell types relevant to tissue engineering applications could be co-cultured in the composite constructs via encapsulation of one cell population, pancreatic islets, in the PEG hydrogel component and a second population, vascularization-promoting endothelial cells, within the fibrin ribbons.

Materials and Methods

Scaffold formation

Photopolymerizable PEG macromers were synthesized as previously described^{16,17}. For unmodified PEG hydrogels, a 7.5wt% PEG macromer solution was photopolymerized under a 365nm ultra-violet light ($\sim 4\text{mW}/\text{cm}^2$) using 0.025wt% photoinitiator (Irgacure-2959). Hydrogels with dispersed fibrinogen were fabricated by mixing thrombin and fibrinogen (Sigma, St. Louis, MO) to final concentrations of 0.5units/mL and 83mg/mL, respectively. In several studies, a small amount (6vol% of fibrinogen solution) of fluorescently-labeled fibrinogen (Invitrogen, Carlsbad, CA) was added to aid in fibrin visualization. The thrombin/fibrinogen solution (15 μL total) was immediately dispersed in 45 μL of 7.5wt% PEG macromer and photopolymerized without allowing time for significant fibrin clotting.

Composite hydrogel constructs with encapsulated fibrin ribbons were formed by mixing thrombin and fibrin as described above and incubating the solution at 37°C for 10 min, allowing time for fibrin ribbon formation. Low concentrations of thrombin and high concentrations of fibrinogen were selected to promote local fibril formation without broad initiation and clotting of the entire mixture¹⁸. Fibrin ribbons were then distributed within the PEG macromer solution and were partially aligned by drawing the plunger of the cutoff syringe in which the scaffold formation took place downward. The resulting fibrin ribbons were then photoencapsulated as described above.

The fibrin ribbon formation and encapsulation protocol described above was modified slightly for experiments in which human umbilical vein endothelial cells (HUVECs) were the encapsulated cell population. In these experiments, final concentrations of thrombin, fibrinogen, and cells within this solution were 83mg/mL, 2.5units/mL, and 3×10^6 cells/mL, respectively. This mixture was incubated for 5 min at room temperature before resulting fibrin ribbons were dispersed in PEG macromer and photopolymerized. The fibrin ribbon forming protocol used for both cell-free ribbons and fibroblast-loaded ribbons did not lead to well-defined fibrin ribbons when endothelial cells were present, necessitating higher levels of thrombin. This phenomenon could be the result of either: (1) direct inhibitors of thrombin (e.g. thrombomodulin and protease nexin-1)¹⁹ or (2) fibrinolytic enzymes^{20,21}, both of which are expressed by endothelial cells. In these experiments, when thrombin concentrations were increased, well-formed fibrin ribbons indistinguishable in size and distribution from those formed in our other scenarios were generated.

Fibroblast culture

3T3 fibroblast cells were grown in monolayer prior to encapsulation and maintained at 37°C/5% CO₂ in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.5 $\mu\text{g}/\text{ml}$ fungizone. All media components were purchased from Invitrogen. Cells used were between passage 5 and 10. Prior to encapsulation, fibroblast monolayer was dissociated with 1X trypsin, centrifuged at 1000rpm for 10 min,

and resuspended in fresh culture medium. Fibroblasts were subsequently mixed with thrombin and encapsulated in fibrin fibrils at a concentration of 5×10^6 cells/mL.

Endothelial cell culture

HUVECs were grown in monolayer prior to encapsulation and were maintained at 37°C/5% CO₂ in endothelial cell specific Medium-200 containing low serum growth supplement (LSGS), 1% penicillin-streptomycin, and 0.5µg/ml fungizone. All media components were purchased from Invitrogen. A single cell suspension of HUVECs was generated as described above for 3T3 fibroblasts. The HUVEC pellet was resuspended in fresh media to a concentration of 6×10^6 cells/mL. HUVECs were utilized after 2–6 cell passages.

For cell seeding experiments, 15µL of the HUVEC cell suspension was carefully dropped onto unmodified PEG hydrogels, hydrogels with dispersed fibrinogen, and composite constructs with encapsulated fibrin ribbons. The cells were allowed to adhere to the gels for 90-min prior to adding additional culture media to the wells.

Visualization of encapsulated and seeded cells

On days 0, 3, and 7 fibroblast and endothelial cells encapsulated within or seeded on top of hydrogel constructs were visualized using a LIVE/DEAD® fluorescent probe membrane integrity assay (viable and dead cells are labeled green and red, respectively) (Molecular Probes, Eugene, OR).

Islet isolation and co-culture in composite PEG/fibrin ribbon constructs

Islets from adult Balb/c mice were obtained from the Diabetes and Endocrinology Research Center at the Barbara Davis Center for Childhood Diabetes (Denver, CO) and isolated as previously described²². Islets were gently mixed into a PEG macromer solution which was subsequently used to disperse endothelial cell-loaded fibrin ribbons. Approximately 30 islets were encapsulated in 45µL of macromer solution per composite construct. For co-culture experiments, endothelial cell (HUVEC) suspension was generated as described above with the following exception: in half of the culture flasks, 10µM CellTracker™ Green CMFDA (Invitrogen) was incubated with HUVEC monolayers prior to trypsin dissociation. Endothelial cells, therefore, in half of the resulting co-culture gels fluoresced green and were easily distinguished. Co-cultures were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.5µg/ml fungizone.

Cells encapsulated in co-culture composite hydrogels were visualized using a LIVE/DEAD® fluorescent probe membrane integrity assay both immediately following encapsulation and after 24 hours of culture.

Immunohistochemistry

At 0- and 24-hour time points, composite co-culture hydrogels were fixed in 4% paraformaldehyde followed by dehydration in 15% sucrose (wt/vol.) for immunohistochemical staining. 40µm frozen sections were stained using standard immunohistochemical techniques. Primary and secondary antibodies used were rabbit anti-mouse insulin (1/60, Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa Fluor® 405 or 488 goat anti-rabbit (1/300, Invitrogen), respectively. A DAPI nuclear counterstain was also used in several images.

Statistical analysis

Statistical significance was determined by using two-tailed, unpaired Student's t-test with significance set at $p < 0.05$. >10 images from ≥ 3 constructs were analyzed for all quantitative

results shown. $n \geq 3$ separate experiments were utilized for all described studies except for islet co-culture studies where $n=1$. Data presented as mean $\pm$ SEM unless otherwise noted.

Results

Formation and characterization of PEG hydrogel encapsulated fibrin ribbons

Carefully-timed thrombin/fibrinogen incubations were imperative in forming large fibrin ribbons prior to mixing and distribution within the PEG macromer solution and subsequent composite hydrogel photopolymerization. If thrombin and fibrin were combined and then immediately mixed with the PEG macromer solution, distributed fibrinogen molecules and ill-formed fibrin clumps were present in the final hydrogel (Fig. 1b). If, however, thrombin and fibrin solutions were combined and incubated for 10 minutes prior to the addition of PEG macromer, well-formed fibrils formed and were subsequently distributed throughout the resulting construct (Fig. 1c). Longer incubation times were also tried (>15 minutes); however, this resulted in a single, large fibrin clot (Fig. 1d). The diameter of fibrin ribbons distributed within composite hydrogels was measured from confocal images (e.g. Fig. 1b) and the size distribution was determined (Fig. 1e). Importantly, the majority of fibrin ribbons are large enough to theoretically support vessel growth and development ($>50\mu\text{m}$), a target size previously determined in porous scaffold vascularization¹².

Fibroblast and endothelial cell encapsulation and culture within fibrin ribbons suspended in a PEG hydrogel

Adherent-dependent fibroblasts were combined with thrombin prior to mixing and incubation with fluorescently-labeled fibrinogen. PEG macromer solution was subsequently added to this mixture after 10-minute incubation and fibroblast loaded fibrin ribbons were encapsulated into the composite hydrogel (Fig. 2a,b). Cell-loaded composite constructs were cultured for 7 days, after which the viable fibroblasts could be seen spreading out along the fibrin fibrils (Fig. 2c,d). Endothelial cells were also mixed with thrombin, combined with fluorescently-labeled fibrinogen, and endothelial cell-loaded fibrin ribbons were encapsulated and cultured within PEG hydrogels. Once encapsulated within fibrin ribbons that were distributed throughout the PEG hydrogel, endothelial cells behaved in a similar manner to fibroblasts, with viable cells spreading along and forming multicellular structures lining the fibrin ribbons (Fig. 3c,f,i). In contrast, endothelial cells encapsulated in unmodified PEG hydrogels survived encapsulation, but quickly decreased in viability by days 3 and 7 of culture (Fig. 3a,d,g). This is unsurprising as endothelial cells are adherent dependent and are unable to attach to an unmodified PEG hydrogel. We also encapsulated endothelial cells in PEG hydrogels with dispersed fibrinogen instead of well-formed fibrin ribbons (Fig. 3b,e,h). The inclusion of fibrin allowed for enhanced cell viability compared to unmodified PEG hydrogels; however, the majority of cells remained isolated and did not form multi-cellular connections.

Fibrin ribbon directed penetration of endothelial cells into composite constructs

Unmodified PEG hydrogels, PEG hydrogels with dispersed fibrinogen, and composite structures with fibrin ribbons distributed throughout a PEG hydrogel were seeded with endothelial cells and cultured for 7 days (Fig. 4). Endothelial cells seeded on unmodified PEG hydrogels did not remain attached when the hydrogels were transferred from the culture medium to the imaging well on day 0, and were likewise not visible on the hydrogel surface on day 3 (Fig. 4a,d), consistent with previously published data¹³. When fibrinogen was dispersed within the hydrogel, cells attached to the surface and were able to penetrate small distances into the construct by culture days 3 and 7 (Fig. 4b,e,g). In composite constructs with fibrin ribbons distributed throughout the PEG hydrogel, endothelial cells attached to the surface in distinct patches (Fig. 4c), and formed numerous multicellular

projections penetrating deep within the hydrogel by days 3 (~300 μ m penetration) and 7 (~600 μ m penetration) (Fig. 4f,h).

Quantification of endothelial cell projection penetration depth was carried out by: (1) counting numbers of cell projections and (2) drawing a perpendicular line from the seeded hydrogel surface to the end of the projection (Fig. 5). Cell projections often changed course based on the shape and direction of the fibrin track that the projection was following; however, penetration depths perpendicular to the seeded surface were utilized as a readily comparable measurement. When fibrin ribbons were encapsulated within a PEG hydrogel, significantly more projections were able to penetrate deeper into the composite constructs than hydrogels with distributed fibrinogen.

Co-culture of two distinct cell populations within PEG/fibrin ribbon composite constructs

We first used fibroblasts as a model cell type to verify co-culture potential. For these experiments, fibroblasts were originally mixed with either the PEG macromer solution or the thrombin/fibrinogen solution prior to mixing the two fractions. Confocal images of resulting composite hydrogels were analyzed to determine the propensity of cells originally mixed into one fraction to remain localized in that fraction and not distribute into the other component of the composite structure (Fig. 6, Table 1). Fibrin fibrils were easily visualized by mixing a small amount of fluorescently-labeled fibrinogen solution with unlabeled fibrinogen prior to fibrin ribbon formation and cells were labeled with LIVE/DEAD® fluorescent probe membrane integrity assay (Fig. 6). An outline of the fibrin ribbon was drawn on each image and cells falling inside or outside of that outline were counted to determine if cells remained in their initial fraction (PEG hydrogel or fibrin ribbons) or distributed into both fractions of the composite hydrogels. When originally mixed with PEG macromer or fibrinogen/thrombin solution, 94% and 77% of cells, respectively, remained in the starting component (Table 1). Because fibrin ribbons made up a smaller percentage of the composite hydrogels, it made sense that a greater proportion of cells originally associated with this fraction would migrate into the larger PEG fraction. Based on this inherent inequity, we also calculated theoretical values to quantify the number of cells that did not mix into the other fraction of the composite hydrogel, but would have if random distribution had occurred (i.e., if cells could freely move into both components of the hydrogel during the mixing required to distribute the fibrin fibrils into the PEG macromer; Table 1). Theoretical values for the propensity of cells to remain in their original component were similar between cells starting in fibrin or PEG fraction because these calculations accounted for fractional area differences, with larger fractional areas (i.e., the PEG fraction) predicted to have more cells. This data demonstrated that a strong majority of cells remained in the original component of the composite hydrogel, validating the use of these structures for co-culture of two distinct cell populations.

To demonstrate the ability of our composite structures to support the co-culture of two distinct, and clinically relevant, cell populations, adult murine pancreatic islets were added to the PEG macromer solution into which we then distributed endothelial cell-loaded fibrin ribbons prior to polymerization. Images of resulting co-cultures taken 24 hours after formation show viable islets, stained with an antibody directed against insulin, suspended in PEG hydrogel surrounded by fibrin ribbons with encapsulated endothelial cells (Fig. 7). To readily distinguish the two cell populations in hydrogel sections, endothelial cells in half of the co-cultures were incubated in a cell tracker solution prior to encapsulation (Fig. 7b,c). In the remaining co-cultures, insulin negative endothelial cells are seen in the fibrin ribbons via nuclear stain (Fig. 7d,e). The average distance from an islet to an endothelial cell-loaded fibrin ribbon, measured from confocal images, was 69 μ m, with 85% of islets (22/26) located within 100 μ m.

Discussion

Here we describe the development and characterization of a novel, three-dimensional composite scaffold containing large fibrin ribbons distributed throughout a PEG hydrogel. We demonstrated that encapsulated fibrin ribbons support the culture of two adherent-dependent cell populations, vascular endothelial cells and fibroblasts. Additionally, by providing permissive fibrin tracts for adherent-dependant endothelial cell penetration our composite scaffolds promoted the infiltration of a PEG hydrogel with multicellular projections of seeded vascular endothelial cells. And finally, we demonstrated our ability to culture two distinct cell populations within the composite constructs by encapsulating endothelial cells within the fibrin ribbons and pancreatic islets in the surrounding PEG hydrogel. Importantly, in these co-culture experiments, the average separation between islets and an endothelial cell-loaded fibrin ribbon was $\sim 70\mu\text{m}$, indicating that our composite constructs have the potential to bring an oxygen supply into close proximity of hydrogel encapsulated and hypoxia sensitive islets.

Using this composite construct of fibrin ribbons distributed in a non-adhesive PEG hydrogel, we showed that endothelial cells encapsulated in fibrin ribbons form organized, multicellular structures lining the fibrin tracts. The ability to develop a network of endothelial structures *in vitro*, provides the potential to develop anastomoses and rapid construct vascularization once implanted *in vivo*. Rapid implant vascularization is of particular importance when delivering hypoxia-sensitive tissues such as islets. Hypoxia can lead to islet cell necrosis and functional deficiencies and is partially responsible for the large number of transplanted islets needed to achieve insulin independence²³. Two findings highlight the utility of transplanted endothelial cells in promoting implant vascularization: (1) donor endothelial cells transplanted along with freshly isolated islets in mouse models anastomose with penetrating host endothelial cells facilitating islet revascularization¹⁴, and (2) prevascularized porous networks implanted *in vivo* demonstrate more rapid perfusion of the implant via inosculation between host vasculature and implanted endothelial cell structures¹⁵. We have demonstrated that islets and endothelial cells can be co-cultured within our composite hydrogels and these prior studies suggest that transplanting endothelial cells along with functioning target cells may help encourage more rapid scaffold vascularization. Interestingly, additional research has shown that co-encapsulating HUVECs and fibroblasts together within a fibrin scaffold significantly enhanced microvessel formation *in vitro* and increased the number and size of vessels formed, as well as the speed of anastomosis and vessel perfusion, after *in vivo* implantation²⁴. We have shown that both fibroblast and endothelial cells thrive when encapsulated within fibrin ribbons, providing a platform for future explorations into the co-encapsulation of these cell types within fibrin ribbons to further enhance *in vitro* development of capillary-like structures and the potential for *in vivo* vascularization of our PEG hydrogel-based scaffolds.

Porous materials have been extensively explored for the purpose of scaffold vascularization. Biocompatible and implantable porous scaffolds have been generated from materials such as alginate, chitosan/collagen, PLG, poly(lactic-co-glycolic acid) (PLGA), and even PEG; however, cells cannot be physically encapsulated within these porous devices due to cytotoxic steps used in their formation^{11,12,25–27}. Porous scaffolds rely instead on post-fabrication cell seeding to deliver the target cell type, which is not ideal for delivery of self-associating and relatively non-adherent three-dimensional microtissues such as islets^{28,29}. Furthermore, surface seeded target cells would remain exposed to attacking immune cells. In our PEG/fibrin ribbon composite scaffolds the target cell type and the fibrin ribbons are distributed within the PEG macromer solution and subsequently photoencapsulated within the resulting hydrogel under cytocompatible conditions. Furthermore, we have demonstrated

that adult murine islets survive composite hydrogel formation and can be encapsulated within the PEG hydrogel fraction of our composite gels.

Our *in vitro* results demonstrate that endothelial cells seeded on the surface of PEG/fibrin ribbon scaffolds are able to form multicellular endothelial cell projections that penetrate deep (~600µm on average) into the construct along the fibrin ribbons after only one week in culture. Additionally, the number of penetrating endothelial cell projections (12 projections/mm²) is comparable to the capillary density of a porous alginate scaffold after 10 and 21 days of *in vivo* implantation¹². Interestingly, it has been demonstrated that filling a porous scaffold with fibrin significantly enhanced functional vessel formation *in vivo*; suggesting that a permissive material for endothelial cell attachment and migration is beneficial over open pores^{25,30}. Taken together, these studies coupled with our *in vitro* data support the inclusion of permissive fibrin ribbon tracks into our hydrogel as a promising method for scaffold vascularization. Within fibrin gels, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been utilized alone and in combination to increase vascularization^{31,32}. In future studies, these growth factors could be incorporated into PEG/fibrin ribbon composite scaffolds to further encourage endothelial cell growth, penetration, and capillary formation.

In summary, we report the development of a composite construct containing fibrin ribbons distributed within a PEG hydrogel. We have shown that adherent-dependent cell populations can be cultured within PEG hydrogel encapsulated fibrin ribbons. Furthermore, we demonstrated that pancreatic islets and endothelial cells, two distinct and clinically relevant cell populations, can be co-cultured via encapsulation in the PEG and fibrin fractions of the composite structure, respectively. These scaffolds also have vascularization potential as demonstrated by the penetration of multicellular endothelial cell projections into the hydrogel along fibrin ribbons. Future studies designed to further explore the utility of the PEG/fibrin ribbon scaffolds will include *in vivo* implantation of composite hydrogels both with and without endothelial cells and/or angiogenic factors incorporated within the fibrin ribbon component. Engineered tissues require efficient oxygen, nutrient, and waste transport and therefore scaffold vascularization is an important step toward creating functional and integrated tissue engineered constructs. PEG hydrogels are advantageous in that they provide a mechanically stable, immunoprotective barrier between immune cells and the encapsulated cells, in addition to being a non-adhesive, bioinert blank slate upon which specific modifications can be made to optimize viability and function of encapsulated cells. Our composite construct provides a potential mechanism for the vascularization of a well-established biomaterial without inherently changing its desirable properties.

Acknowledgments

This project was supported by Award Number VUMC35144 from the Beta Cell Biology Consortium, by Award Number F30DK081278 from the National Institute Of Diabetes And Digestive And Kidney Diseases, and by the Department of Education's Graduate Assistantships in Areas of National Need Program. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute Of Diabetes And Digestive And Kidney Diseases or the National Institutes of Health. The authors would also like to thank Philip Pratt at the Barbara Davis Center for Childhood Diabetes for the islet isolation.

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Figure 1.

Fibrin ribbon formation and distribution within PEG hydrogels. Fluorescently-labeled fibrinogen was added to the fibrinogen/thrombin solution enabling fibrin visualization. Images of an unmodified PEG hydrogel (a) and of PEG hydrogels with fibrinogen/thrombin solution distributed immediately after mixing (b), and after a 10-min (c) or 15-min (d) incubation at 37°C are shown. (b) Without the required incubation time, fibrils do not form and fibrin is dispersed in the PEG hydrogel as unstructured clumps. (c) With a 10-min incubation, well-formed fibrin ribbons are distributed throughout the PEG hydrogel matrix. (d) If incubation time is increased further to 15-minutes, a single large clot of fibrin is suspended in the PEG hydrogel. Scale bar for (a–c), 50μm. Scale bar for (d), 200μm. (e) For well-formed fibrin ribbons, such as those shown in (c), fibrin ribbon diameters were measured, and the percent of total fibrils falling between the sizes indicated on the x-axis were determined.

Figure 2.

Fibroblast distribution along fibrin ribbons encapsulated in a PEG hydrogel. Fibroblasts initially encapsulated in fibrin fibrils on days 0 (a,b) and 7 (c,d). Fibroblasts demonstrate robust viability, and elongate and distribute along the length of fibrin fibrils after 1 week in culture. Scale bar, 50 μ m.

Figure 3.

Endothelial cells align when encapsulated within well-formed fibrin ribbons. HUVECs were encapsulated in unmodified PEG hydrogels (a,d,g), fibrinogen/thrombin that was immediately mixed with PEG macromer solution precluding fibril formation (b,e,h), and fibrin ribbons encapsulated in a PEG hydrogel (c,f,i). Cultures were stained with a LIVE/DEAD® assay on days 0 (a,b,c), 3 (d,e,f), and 7 (g,h,i) and are shown as a series of projected images taken with a confocal microscope. A small amount of fluorescently-labeled fibrin was added to the fibrinogen/thrombin solution to visualize the fibrin fibrils in (c,f,i). The punctate distribution of fibrin when dispersed throughout the PEG looked like scattered dead cells and therefore fibrin labeling was not performed for (b,e,h). After 7 days in culture, HUVECs do not remain viable in non-adhesive PEG hydrogels (g) and remain viable but appear mostly as single cells in PEG gels with dispersed fibrin (h). When encapsulated within fibrin ribbons, endothelial cells elongate and form multicellular structures lining the fibrin fibrils (i). Inset in (i) shows more clearly the multicellular structures formed by interconnecting HUVEC cells without the fibrin fibril labeling. Scale bar, 100µm.

Figure 4.

Endothelial cells penetrate into PEG hydrogels along encapsulated fibrin ribbons. HUVECs were seeded on top of unmodified PEG hydrogels (a,d), PEG hydrogels with dispersed fibrin (b,e,g), and PEG hydrogels with encapsulated fibrin fibrils (c,f,h). Cultures were stained with a LIVE/DEAD® assay on days 0 (a,b,c), 3 (d,e,f), and 7 (g,h) and are shown as a series of projected images taken with a confocal microscope. HUVECs were unable to sufficiently adhere to the surface of PEG hydrogels to remain seeded when transferring the gel from the culture well to the imaging well (a). Likewise, no cells were visualized on the surface of unmodified PEG hydrogels on days 3 (d) or 7 (data not shown). Cells adhered to PEG hydrogel surfaces with dispersed fibrin and showed small penetrating projections on both days 3 (e) and 7 (g). Endothelial cells seeded on the surface of PEG gels with encapsulated well-formed fibrin ribbons adhere on day 0 (c), show multiple penetrating projections on day 3 (f), and an extensive network of penetrating, multicellular endothelial cell projections by day 7 (h). A view of the top of a seeded PEG/fibrin ribbon hydrogel shows the clustering of seeded endothelial cells in distinct patches where fibrin ribbons are located (c. inset). A fluorescently labeled fibrin fibril (red) with DAPI-labeled penetrating HUVECs (blue) is shown as an inset to (f), verifying fibrin ribbon directed penetration. Scale bar, 100µm.

Figure 5. Quantification of seeded endothelial cell penetration into PEG hydrogels with dispersed fibrin and with well-formed fibrin ribbons. (a–c) The number (a), average length (b), and the average of the maximum length (c) of multicellular, endothelial cell projections into PEG hydrogels with dispersed fibrin (white bars) and with well-formed fibrin fibrils (black bars) were determined after 3 and 7 days of culture. Values in (a) are presented as number of projections per mm² of seeded surface area and in (b) and (c) as µm of penetration. All values were obtained by counting the number and measuring the length of projections from LIVE/DEAD® images, such as those shown in Figure 6. Values for unmodified PEG hydrogels were 0 for all quantities measured (not shown). ** $p < 0.01$ and *** $p < 0.001$ compared to time-matched dispersed fibrin hydrogels.

Figure 6.

Series of confocal images depicting how cell counts were conducted to determine values shown in Table 1. 3T3 fibroblasts were used as a model cell type to demonstrate our ability to restrict a cell type to either the fibrin fibril fraction (a–c) or the PEG fraction (d–f) of the composite hydrogels, depending on which component the cells were added to prior to mixing of PEG macromer and fibrinogen/thrombin solutions. Single images were taken after hydrogel polymerization and calcein-AM staining of live cells green. Fluorescently-labeled fibrinogen was added to the fibrinogen/thrombin solution so that fibrin fibrils would appear red. Top images show 3T3s mixed with fibrinogen/thrombin solution (a–c) and bottom images show 3T3s mixed in the PEG macromer solution (d–f). In both conditions, the fibrin fibril shown in the image was outlined with the green channel turned off (b,e). Cells within and outside of the outlined fibril were subsequently counted (c,f).

Figure 7.

Co-culture of pancreatic islets and endothelial cells in composite PEG/fibrin ribbon hydrogels. (a) Image showing fibrin ribbons (red) with encapsulated endothelial cells near a pancreatic islet encapsulated in the PEG hydrogel fraction of the composite structure. Live cells (a; both islet and endothelial cells) are labeled green with a calcein-AM dye. Scale bar for (a), 50 μ m. (b,c) Immunohistochemical images showing pancreatic islets stained for insulin (purple), fibrin ribbons (red), and endothelial cells labeled with a cell tracking dye prior to encapsulation (green). (d,e) For images where endothelial cells were not pre-labeled, insulin staining (green), cell nuclei (purple), and fibrin ribbons (red), are shown. Scale bar for (b–e), 20 μ m.

Table 1

The propensity of cells to remain in the composite hydrogel fraction into which they were initially mixed.

			Composite hydrogel fractions	
			PEG	Fibrin
% of cells in initial fraction			100%	100%
Measured values	% of cells that...	remained in the initial fraction	94 ± 1.4%	77 ± 5.2%
		moved to the other fraction	6%	23%
Theoretical values *	% of cells predicted to move...	but they did not change fractions	76 ± 4.3%	74 ± 5.8%
		and they did change fractions	24%	26%

* Based on the fractional area of each hydrogel fraction (PEG vs. Fibrin) and random distribution of encapsulated cells