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Journal of Biomaterials Science 20 (2009) 1763–1779

Journal of BIOMATERIALS SCIENCE
www.brill.nl/jbs

Covalently-Immobilized Vascular Endothelial Growth Factor Promotes Endothelial Cell Tubulogenesis in Poly(ethylene glycol) Diacrylate Hydrogels

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Received 29 July 2008; accepted 16 October 2008

Abstract

The development and use of functional tissue-engineered products is currently limited by the challenge of incorporating microvasculature. To this end, we have investigated strategies to facilitate vascularization in scaffold materials, in this case poly(ethylene glycol) (PEG) hydrogels. These hydrogels are hydrophilic and resist protein adsorption and subsequent non-specific cell adhesion, but can be modified to contain cell-adhesive ligands and growth factors to support cell and tissue function. Additionally, the hydrogel matrix can include proteolytically degradable peptide sequences in the backbone of the structure to allow cells to control scaffold biodegradation, allowing three-dimensional migration. Vascular endothelial growth factor (VEGF), a potent angiogenic signal, and the cell-adhesive peptide RGDS were each covalently attached to PEG monoacrylate linkers. PEGylated RGDS and VEGF were then covalently immobilized in PEG-diacrylate (PEGDA) hydrogels in 2D and 3D. Immobilized VEGF increased endothelial cell tubulogenesis on the surface of non-degradable PEGDA hydrogels 4-fold compared to controls without the growth factor. Endothelial cell behavior in 3D collagenase-degradable hydrogels modified with RGDS and VEGF was observed using time-lapse confocal microscopy. Bulk immobilization of VEGF in 3D collagenase-degradable RGDS-modified hydrogels increased endothelial cell motility 14-fold and cell–cell connections 3-fold. Covalent incorporation of PEGylated VEGF in PEG hydrogels can be a useful tool to promote endothelial cell migration, cell–cell contact formation and tubulogenesis in an effort to produce vascularized tissue-engineered constructs.
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Keywords

Angiogenesis, hydrogel, PEG, VEGF

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© Koninklijke Brill NV, Leiden, 2009 DOI:10.1163/156856208X386381

1. Introduction

The induction of microvasculature into engineered tissues is currently a roadblock to successfully engineering functional tissues of significant thicknesses. Recent clinical successes in tissue engineering, including skin [1, 2] and bladder [3], are feasible because these tissues are thin and can rely on diffusion for transport of nutrients, oxygen and waste. *In vivo*, cells are generally within approx. 100 μm of a blood vessel to allow for efficient transport [4]. Microvasculature is required for tissues thicker than 200 μm [5], otherwise cells undergo necrosis due to low nutrient and oxygen supply in addition to build-up of waste products as a result of the diffusion limits of these molecules in tissue. Because most engineered tissues cannot depend solely on diffusion for transport, microvascularization before implantation should be considered a requirement for three-dimensional engineered tissues. We are interested in whether *in vitro* microvascularization of engineered tissues may be achieved by mimicking the natural *in vivo* process of capillary formation, angiogenesis. During angiogenesis, endothelial cells form capillary networks to allow transport of nutrients, oxygen and waste throughout the tissue. In this process, endothelial cells respond to biochemical angiogenic signals such as vascular endothelial growth factor (VEGF) to form endothelial tubes, which are later stabilized by mural cells. Signaling by VEGF, a protein dimer, is considered a rate-limiting step in the initiation of angiogenesis [6]. We hypothesize that by sequestering and presenting VEGF in an extracellular matrix-like environment, we can promote endothelial cell tubulogenesis.

Sequestering growth factors in a matrix, rather than local release, provides a more engineered and controllable environment and consequent biological response. Release of VEGF has the potential to yield unwanted, non-local activity. Mazue *et al.* showed that too much growth factor can be toxic to red blood cell production and kidneys, and nonlocal growth factor could support tumor growth [7–9]. Additionally, when administered in bolus injections, VEGF is eliminated quickly, with a half-life of less than 1 h [10]. Because of the often unwanted responses due to released growth factors and the biological need for signaling duration not met by bolus release, we have chosen to covalently bind VEGF to a matrix that supports cell adhesion and angiogenic activity.

Synthetic polymer matrices are promising substrates for tissue engineering matrices as they are often mechanically stronger and more easily characterized than naturally-derived materials. Poly(ethylene glycol) diacrylate (PEGDA) is a biocompatible, hydrophilic polymer that can be cross-linked to form hydrogels with tunable mechanical properties [11, 12]. Biocompatible photoinitiators can be utilized for crosslinking [11, 13, 14]. PEGDA resists protein adsorption and subsequent non-specific cell adhesion and, thus, acts as a “blank slate” for designing an approximate biological environment to guide complex tissue organization [11]. PEGDA-based hydrogels can be modified with cell-adhesive ligands, bioactive growth factors and proteolytically degradable peptide sequences to generate bioactive hydrogels [12, 15]. Proteolytically degradable PEG derivatives allow 3D culture of cells encapsu-

lated in the matrix as well as cell migration into the matrix, which occurs when cells secrete proteases, cleaving the collagenase-degradable peptide backbone [16].

In the current studies, PEG-based hydrogels were modified to incorporate local, controlled biochemical signaling to cells. VEGF was covalently coupled to the PEGDA hydrogel matrix, and the endothelial cell responses to the sequestered growth factor were analyzed. A synthetic matrix was developed which can support and promote *in vitro* tubulogenesis, the first step in creating a functional microvasculature in engineered tissues.

2. Materials and Methods

2.1. Cell Culture

Human umbilical vein endothelial cells (HUVEC, Cambrex/Lonza, Walkersville, MD, USA) were used between passages 2 and 6. Cells were maintained in VEGF-free endothelial cell growth medium (EGM-2 media, Cambrex/Lonza) that contained hydrocortisone, fibroblast growth factor (hFGF-B), insulin-like growth factor (R^3-IGF-1), ascorbic acid, epidermal growth factor (hEGF), GA-1000 (gentamicin, amphotericin-B), heparin, 2% fetal bovine serum (FBS) (Bulletkit, Lonza), 2 mM L-glutamine, 1 U/ml penicillin and 1 μg/ml streptomycin (GPS, Sigma, St. Louis, MO, USA). Cells were maintained at 37°C in a 5% CO_2 environment. Before 3D encapsulation, HUVECs were labeled with Celltracker Green CMFDA (Molecular Probes, Eugene, OR, USA). 50 μg Celltracker Green was dissolved in 5 μl DMSO and diluted in cell media for a final concentration of 2 μg/ml. HUVECs were incubated in the prepared media for 1 h. Labeling was visually confirmed, and labeling media was replaced with normal EGM-2 (without VEGF). Cells were washed repeatedly with phosphate-buffered saline (PBS) before enzymatic lifting using trypsin and subsequent encapsulation.

2.2. Preparation and Purification of Poly(ethylene glycol) Diacrylate (PEGDA)

Poly(ethylene glycol) (PEG, 6000 Da; Fluka/Sigma) was reacted with acryloyl chloride (Sigma) at a 1:4 molar ratio in anhydrous dichloromethane (DCM; Sigma) with triethyl amine (TEA; Sigma) at a 1:2 (PEG/TEA) molar ratio under argon overnight at 25°C (Fig. 1A). PEGDA was purified *via* phase separation using 2 M K_2CO_3. The organic phase containing PEGDA was dried using anhydrous $MgSO_4$ and filtered. PEGDA was precipitated in diethyl ether, filtered, and dried overnight and under vacuum, then characterized by ^{1}H-NMR and stored at −20°C under argon until use.

2.3. Preparation and Purification of PEG-Succinimidyl Carbonate (PEG-SMC)

Due to a discontinuation of Acryloyl-PEG-NHS (Nektar, Huntsville, AL, USA) to the research community, PEG-SMC, which has similar chemical functionality to PEG-NHS, was synthesized. Monoacrylated PEG was prepared by reacting PEG (3400 Da; Fluka/Sigma) with 1.5 molar excess Ag_2O (Sigma), 1.1 molar

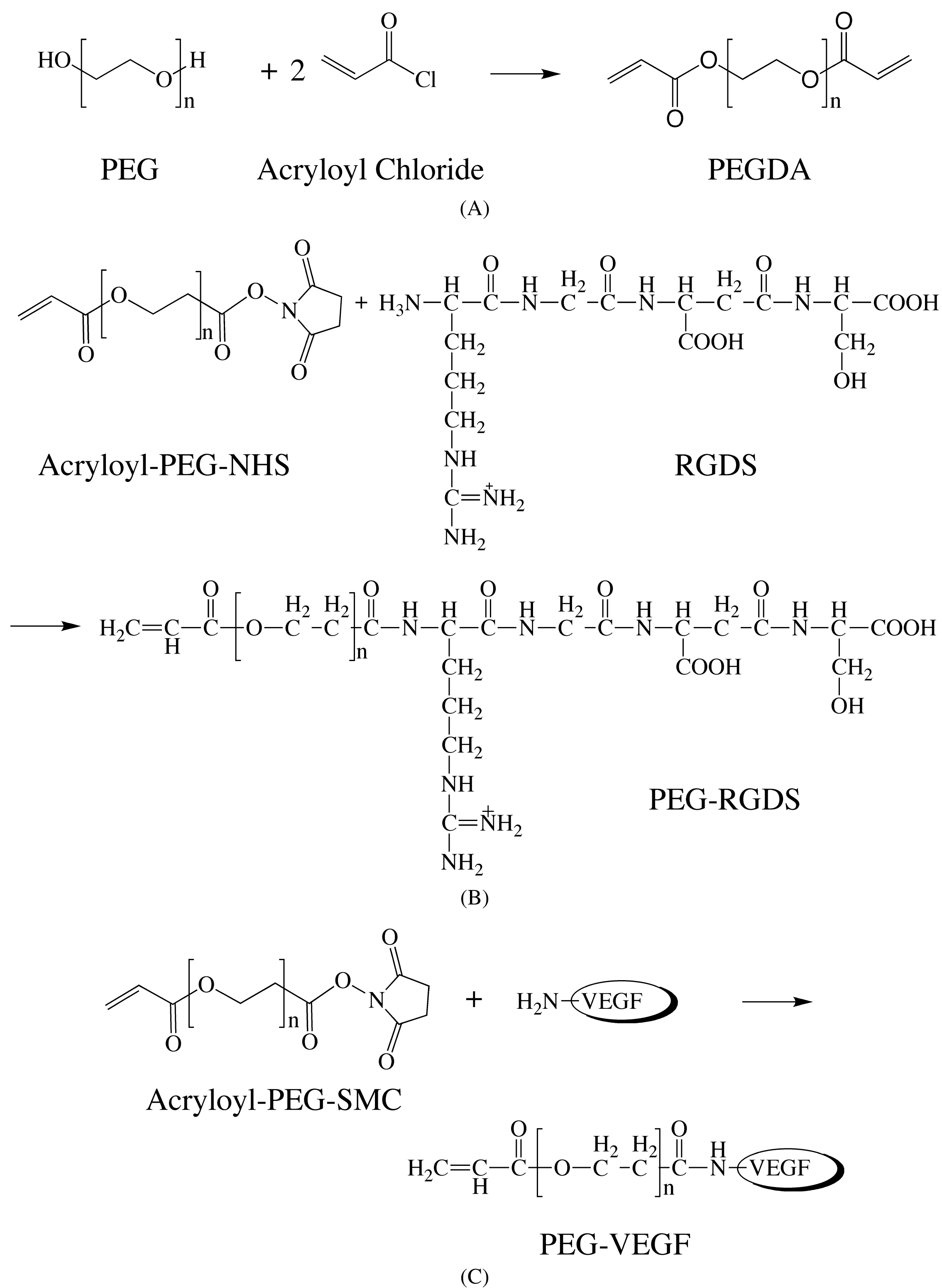


Figure 1. (A) Chemical synthesis of poly(ethylene glycol) diacrylate, (B) PEGylation of adhesive peptide RGDS and (C) PEGylation of angiogenic protein VEGF.

excess acryloyl chloride (Sigma) and 0.3 molar ratio KI (Sigma) in anhydrous dichloromethane (DCM; Sigma) overnight at 0–4°C. The resulting solution was filtered using Celite 521 (Spectrum Chemical Manufacturing, Gardena, CA, USA) to remove silver. The filtered solution was dried using a Rotovap, and then dissolved in DI H_2O and the pH was altered to pH 3 using HCl. The solution was then heated to 35°C for 1 h. Activated charcoal (Fisher) was added to the mixture to remove iodine. The solution was subsequently filtered using Celite 521. NaCl was added with DCM, followed by DCM extraction. Chloride ions and acid were removed *via* phase separation using 2 M K_2CO_3. Monoacrylated PEG was dried using sodium sulfate (Fisher Scientific, Pittsburgh, PA, USA), concentrated using a Rotovap, precipitated in ethyl ether, and vacuum filtered. PEG-monoacrylate was then reacted with 4 molar excess disuccinimidyl carbonate (Sigma) in anhydrous acetonitrile (Sigma) and pyridine (Sigma) under argon overnight. The product was dried using a Rotovap, dissolved in anhydrous DCM, and filtered. PEG-SMC was isolated *via* phase separation in acetate buffer (0.1 M, pH 4.5, 15% NaCl), dried using anhydrous $MgSO_4$, filtered, precipitated in ethyl ether, filtered, and dried overnight and under vacuum. PEG-SMC was characterized by ^{1}H-NMR and MALDI-TOF and stored at −80°C under argon until use.

2.4. Preparation and Purification of PEG-RGDS

The cell-adhesive peptide RGDS (American Peptide, Sunnyvale, CA, USA) was dissolved in 50 mM sodium bicarbonate buffer (pH 8.5) at a concentration of 25 mM. Acryloyl-PEG-N-hydroxysuccinimide (PEG-NHS, 3400 Da; Nektar) or PEG-SMC was similarly dissolved at a concentration of 50 mM. PEG-NHS or PEG-SMC was added drop-wise to RGDS in a 1:1 molar ratio with slow mixing and allowed to react for 2 h at 25°C or 4 days at 4°C, respectively. The product was dialyzed against DI H_2O for 8 h using a membrane with a 1 kDa molecular mass cutoff (Spectrum Laboratories, Rancho Dominguez, CA, USA). PEG-RGDS (Fig. 1B) was then lyophilized and stored at −80°C under argon until use. Conjugation was characterized by gel-permeation chromatography (GPC) using a PLgel column (5 μm, 500 Å, Polymer Laboratories, Amherst, MA, USA), 0.1% ammonium acetate in DMF solvent, and evaporative light scattering (ELS) detector (Polymer Laboratories), run against PEG standards.

2.5. Synthesis of Collagenase-Degradable PEG-CSP-PEG

A collagenase-sensitive peptide (CSP) GGGPQGIWGQGK was prepared on a peptide synthesizer (Aapptec, Louisville, KY, USA) using standard Fmoc chemistry. The peptide was cleaved from the resin using 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS) in water and precipitated in ether. The peptide was reacted with acryloyl-PEG-NHS (Nektar) or PEG-SMC in sodium bicarbonate buffer (pH 8.5, 50 mM) to generate a PEG-diacrylate derivative with the CSP in the polymer backbone, and conjugation was confirmed *via* GPC with ELS detection as described above.

2.6. Synthesis of PEG-VEGF

$VEGF_{165}$ (Sigma) was dissolved in sterile 50 mM sodium bicarbonate buffer (pH 8.5, 0°C). Acryloyl-PEG-SMC was similarly dissolved and sterilized *via* filtration (0.2 μm). PEG-SMC was added to VEGF in a 200:1 molar ratio with slow mixing under sterile conditions and allowed to react for 4 days at 4°C, yielding PEG-VEGF. PEG-VEGF (Fig. 1C) was then lyophilized under sterile conditions and stored in HEPES-buffered saline (HBS; 100 mM NaCl, 10 mM HEPES in deionized water; pH 7.4) with 0.1% bovine serum albumin (BSA) at 4°C until use. Conjugation was confirmed *via* Western blot using reducing conditions on a Tris-HCl pre-cast polyacrylamide gel (Bio-Rad, Hercules, CA, USA), rabbit polyclonal anti-VEGF primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), HRP-conjugated goat anti-rabbit IgG (MP Biomedicals, Aurora, OH, USA) and ECL™ chemiluminescent Western blotting analysis system (GE Healthcare, Slough, UK). Film (Kodak, Rochester, NY, USA) was exposed to Western blot membrane for 5 s and then developed using a Micromax Developer (Hope, Warminster, PA, USA) with T_2 developer and T_2 fixer (White Mountain Imaging, Salisbury, NY, USA).

2.7. Formation of PEGDA Hydrogels

PEGDA (6 kDa) was dissolved in HBS (10%, w/v) solution and sterile filtered. 10 μl/ml of 300 mg/ml 2,2-dimethoxy-2-phenylacetophenone in N-vinylpyrrolidone (NVP) was added to the solution. Molds were constructed by placing poly(tetra fluoroethylene) (PTFE, 0.5 mm thickness) spacers between two glass slides on three sides and securing with clips. The polymer solution was pipetted into molds and cross-linked through exposure to long wavelength ultraviolet light (B-200SP UV lamp, UVP, 365 nm, 10 mW/cm^2) for 30 s. After cross-linking, the mold was removed, and the PEGDA hydrogel slab was placed in sterile PBS with 0.1% sodium azide until further use.

2.8. Surface Modification of PEGDA Hydrogels

Hydrogel slabs were soaked for 1 h in sterile PBS to remove sodium azide. Circles, 5 mm in diameter, were punched from PEGDA hydrogel slabs. A polymer solution consisting of 0.42 nmol/ml PEG-VEGF, 30 μmol/ml PEG-RGDS, 1 μmol/ml eosin Y and 3.95 μl/ml NVP was prepared. From this solution, 10 μl was pipetted onto the top surface of the gel, completely covering the surface. The gel and polymer solution were exposed to a 532 nm laser at 30 mW/cm^2 for 30 s. The surface-modified gel was then soaked in sterile PBS for 1 day to allow non-reacted polymer and excess photoinitiator to diffuse from the gel.

2.9. Quantification of Surface-Immobilized VEGF and RGDS

An ELISA assay was used to determine the amount of VEGF that was covalently immobilized on hydrogel surfaces. Briefly, gels were modified with PEG-VEGF and PEG-RGDS, then allowed to soak in HBS with 0.1% BSA for 3 days, with

collection and replacement of the saline solution on the second day. The amount of PEG-VEGF in the saline in samples from both days was quantified using a VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA) with PEG-VEGF standards. The amount of PEG-VEGF released from the gels after 2 and 3 days was then used to calculate surface conjugation.

To quantify the amount of covalently-linked PEG-RGDS on the surface of the gels, a ninhydrin assay was used following degradation of the hydrogels and peptides in acid. The ninhydrin assay measures amine content, which results from the presence of the grafted peptide [17]. Samples were soaked after conjugation to allow unbound RGDS to diffuse from the gel. Standards were created by adding known amounts of PEG-RGDS to PEGDA hydrogels. Standards and hydrogels modified with PEG-RGDS were lyophilized, then degraded using 6 M HCl for 3 h at 100°C. HCl was removed using a Rotovap, and samples, RGDS standards and glycine standards were dissolved in 0.1 M sodium citrate buffer (pH 5). Ninhydrin reagent (Sigma) was added, and the samples were boiled for 15 min, centrifuged, and the resulting colored product was read at 570 nm to determine the amount of RGDS on hydrogels.

2.10. Endothelial Tubule Formation

HUVECs were seeded (8.5×10^4 cells/cm^2) onto gels with either PEG-RGDS only or PEG-RGDS and PEG-VEGF covalently attached to the surface. HUVEC tubulogenic response on the gels was monitored and EGM-2 media changed every other day. Three experimental groups were observed: VEGF- and RGDS-modified hydrogels cultured in EGM-2 media (without soluble VEGF, 1% serum), RGDS-modified hydrogels cultured in EGM-2 media (without soluble VEGF, 1% serum), and RGDS-modified hydrogels cultured in EGM-2 media (with soluble VEGF, 1% serum). Images were taken of each entire gel and merged using Photoshop Elements software. Tubules were traced using Adobe Illustrator software, and length of each tubule was calculated in ImageJ software (NIH, Bethesda, MD, USA). The total sum of tubule length per area was calculated for each sample. Data from separate experiments was pooled, and ANOVA followed by Tukey's Least Significant Difference post hoc analysis was performed to determine significant differences between groups. All data are presented as mean ± standard deviation.

2.11. Formation of Three-Dimensional Proteolytically Degradable PEG Hydrogels

Collagenase-degradable hydrogels with encapsulated HUVEC cells (3×10^7 cells/ml, labeled with Celltracker Green) were prepared. Briefly, PEG-CSP-PEG (0.1 g/ml), acryloyl-PEG-RGDS (3.5 μmol/ml) and acryloyl-PEG-VEGF (200 pmol/ml) were mixed with a cell suspension and photo-cross-linked by exposing to long-wavelength UV (365 nm, 10 mW/cm^2) for 9 min, using Irgacure 2959 as the photoinitiator (0.3%, w/v). Control hydrogels were cross-linked without incorporation of PEG-VEGF.

2.12. Time-Lapse Study of Endothelial Tubulogenesis in Three-Dimensional Degradable PEG Hydrogels

Constructs were cultured for 5 h in EGM-2 media without VEGF at 37°C in a 5% CO_2 environment and then transferred to a confocal microscope (Zeiss Live5, Thornwood, NY, USA) with a stage chamber providing a regulated environment (37°C and 5% CO_2). No additional proteolytic enzymes nor protease inhibitors were added to the culture. Z-stack images were collected every hour for 60 h using the Multi Time Series macro (Zeiss), Plan-Apochromat 20× objective with 0.8 numerical aperture, and excitation wavelength = 489 nm, emission bandpass (BP) filter = 500–525 nm and pinhole = 55 μm. Time-lapse movies were analyzed for cell migration and cell–cell contact formation. For cell migration quantification, the movement of 3 randomly selected cells per viewing field was tracked using Zeiss LSM5 Image Browser software, which allows the tracing and quantification of cell paths through timeframe progression. For cell–cell contact formation quantification, the number of all cell–cell contacts formed within the viewing field was counted by timeframe progression using the same software. Data from separate experiments was pooled, and Student's two-tailed, unpaired t-tests were performed to determine significant differences between groups. Representative samples were fixed with 4% formaldehyde in PBS at 29 h, permeabilized with 0.5% Triton X-100 for 30 min, blocked with BSA for 30 min, stained with Alexa Fluor 568-conjugated phalloidin (10 U/ml, Molecular Probes) for 2 h and DAPI (2 μM, Invitrogen, Carlsbad, CA, USA) for 30 min to label cell actin filaments and nuclei, and visualized using confocal microscopy (Zeiss Live5, Plan-Neofluar oil-immersion 40× objective with 1.3 numerical aperture, for phalloidin, excitation = 532 nm, emission BP filter = 560–675 nm; for DAPI, excitation = 405 nm, emission BP filter = 415–480 nm, pinhole = 10 μm) to obtain Z-stack images which were then processed into 3D projections using ImageJ software.

3. Results

3.1. Polymer Characterization

Conjugation of RGDS to acryloyl-PEG-NHS or acryloyl-PEG-SMC was confirmed *via* GPC, which showed the product PEG-RGDS to have the expected molecular mass. Conjugation of PEG-CSP-PEG was similarly confirmed *via* GPC. Conjugation of VEGF to acryloyl-PEG-SMC was confirmed *via* Western blot (Fig. 2). Native VEGF appears in two bands, at 38 and 19 kDa, indicating dimer and monomer species, respectively. The marked increase in molecular mass of PEG-VEGF indicates successful PEGylation of the protein [12].

3.2. Quantification of PEG-VEGF and PEG-RGDS on the Surface of Hydrogels

Hydrogels had an average of 19 ± 0.75 pmol/cm^2 PEG-VEGF on the surface, which corresponds to a conjugation of 82.5% of the PEG-VEGF in the polymer solution.

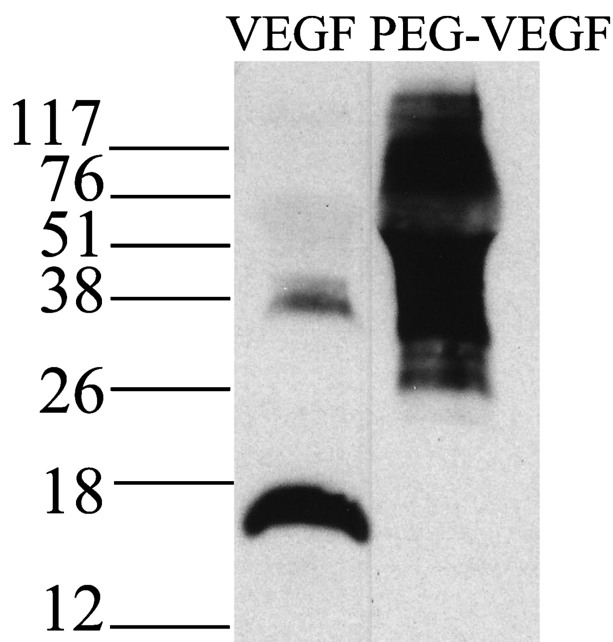


Figure 2. Western blot shows increase in molecular mass after PEGylation of VEGF.

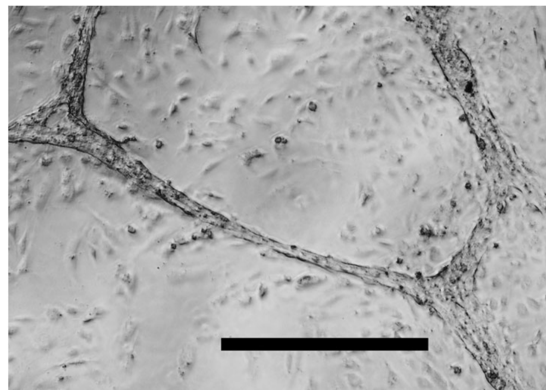
After an additional 24 h incubation in buffer, 98–99% of the initial PEG-VEGF remained attached to the surface. Hydrogels had an average of 5.4 ± 3.2 nmol/cm^2 PEG-RGDS on the surface.

3.3. Surface-Immobilized VEGF Promotes Tubulogenesis

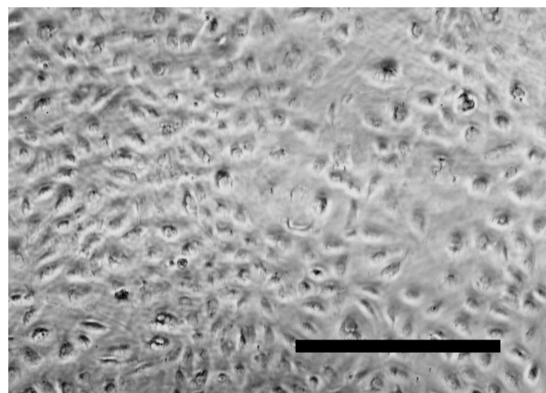
After 30 days in culture, endothelial cells exhibited extensive branching networks of endothelial tubes on hydrogels modified with PEG-VEGF and PEG-RGDS. Control hydrogel surfaces modified with PEG-RGDS allowed HUVEC attachment and growth, but did not promote tubulogenesis. Hydrogels modified with PEG-RGDS and PEG-VEGF promoted significantly more tubulogenesis than those modified with PEG-RGDS alone (Figs 3 and 4; $P < 0.05$). Endothelial cells grown on hydrogels modified with PEG-VEGF and PEG-RGDS, cultured without VEGF in the media, formed tubules totaling 1127 ± 974 μm/mm^2 in length, while cells on hydrogels modified with PEG-RGDS only, cultured without VEGF in the media, formed tubules totaling 234 ± 310 μm/mm^2 in length, and cells on hydrogels modified with PEG-RGDS only, cultured with VEGF in the media, formed tubules totaling 217 ± 591 μm/mm^2. Lower levels of PEG-RGDS on the surface (0.5 and 0.05 nmol/cm^2) did not support extended endothelial cell attachment.

3.4. Immobilized VEGF in 3D Degradable Hydrogels Promotes Cell Motility, Cell–Cell Contact Formation and Tubulogenesis

In 3D PEG hydrogels with immobilized VEGF, endothelial cells exhibited extensive angiogenic behavior, as observed by time-lapse confocal microscopy. Between 21 and 42 h after encapsulation, HUVECs formed elongated multiple-cell structures in hydrogels with RGDS and VEGF homogeneously and covalently bound



(A)



(B)

Figure 3. (A) Branching endothelial tubule networks formed on the surface of hydrogels modified with RGDS and VEGF at 19 days. (B) Fewer tubules formed on hydrogels modified with RGDS only at 19 days. Scale bar = 500 μm.

to the matrix, but less so in hydrogels with RGDS only (Figs 5 and 6). Cells in VEGF-modified hydrogels had significantly more migratory behavior, traveling 14 times farther, and formed 3 times more cell–cell contacts than cells in hydrogels without VEGF (Fig. 7, migration distance $P < 5.45 \times 10^{-7}$, cell–cell contacts $P < 0.006$). Both migration and cell–cell contact formation through surface projections are fundamental behaviors during angiogenesis [18]. Time-lapse confocal microscopy showed endothelial tubes regressing after 51 h, most likely due to the absence of mural cells, such as pericytes, that stabilize forming capillaries. HUVEC migratory behavior in VEGF hydrogels continued until the study ended, suggesting that the covalently-bound VEGF retained bioactivity throughout the study and was able to induce the first steps of angiogenesis: endothelial cell migration, cell–cell contact formation and endothelial tubule formation.

4. Discussion

Several studies have examined releasing or sequestering growth factors in tissue-engineering matrices. Nillesen *et al.* induced *in vivo* capillary formation and matu-

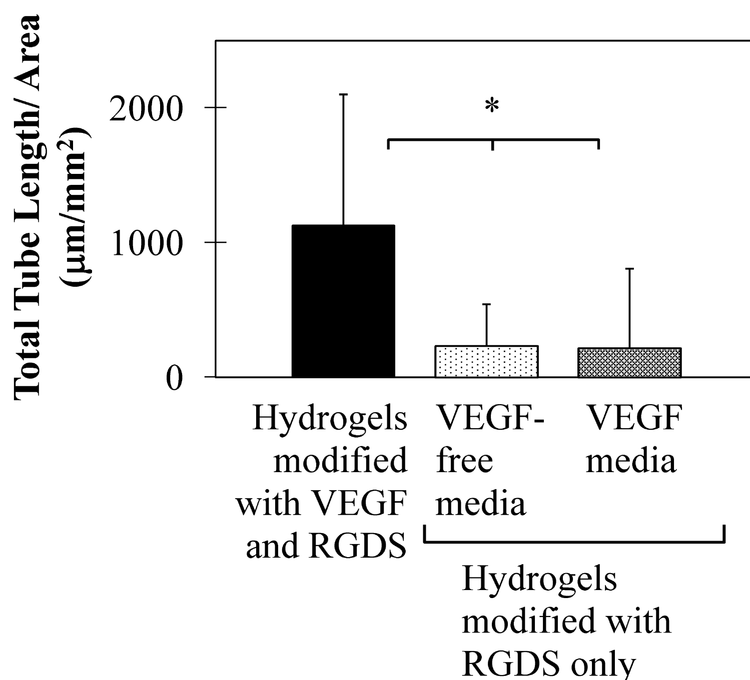


Figure 4. Quantification of tubulogenesis shows a significant endothelial cell response to immobilized VEGF on the surface of PEGDA hydrogels. Error bars show standard deviation (ANOVA $P < 0.01$, Tukey's Least Significant Difference between VEGF group and both RGDS groups $P < 0.05$).

ration in collagen scaffolds by incorporating heparin-bound VEGF and FGF2. The percent of hypoxic cells decreased dramatically from 97–98% at day 3 to 2% by day 7 and 0.2% by day 21 in scaffolds containing VEGF and FGF2 while it remained at 37% (day 7) and 21% (day 21) in collagen scaffolds without growth factors [19]. This study confirms the extended state of hypoxia in non-vascularized tissues and also points to the longevity of signaling and biological response to matrix-bound growth factors. Collagen scaffolds with incorporated heparin, however, allow continual release and rebinding of growth factors, and can interact not only with the desired growth factors, but also with other proteins supplied by the host at the area of the implant. Thus, the environment is not regulated, and the results cannot be shown to be directly related to the influence of the matrix design.

Peters *et al.* engineered a poly(lactide-co-glycolide) (PLG)-Matrigel matrix to control the local release of VEGF. Pores were created in the VEGF-releasing PLG matrix to allow endothelial cell vessel formation, and human microvascular endothelial cells were suspended in Matrigel, which was then absorbed into the polymer scaffold. Human microvascular endothelial cells formed capillaries within the PLG-Matrigel matrix *in vivo* in SCID mice in 5 days [20]. This study relied on released VEGF and Matrigel to promote angiogenesis within a synthetic matrix. The release kinetic profile of VEGF was engineered into the synthetic matrix design; however, the matrix was not used to support cell adhesion on its own. Further engineering and refinement of synthetic matrices incorporating growth factors could improve control of angiogenic cellular behavior both *in vitro* and *in vivo*.

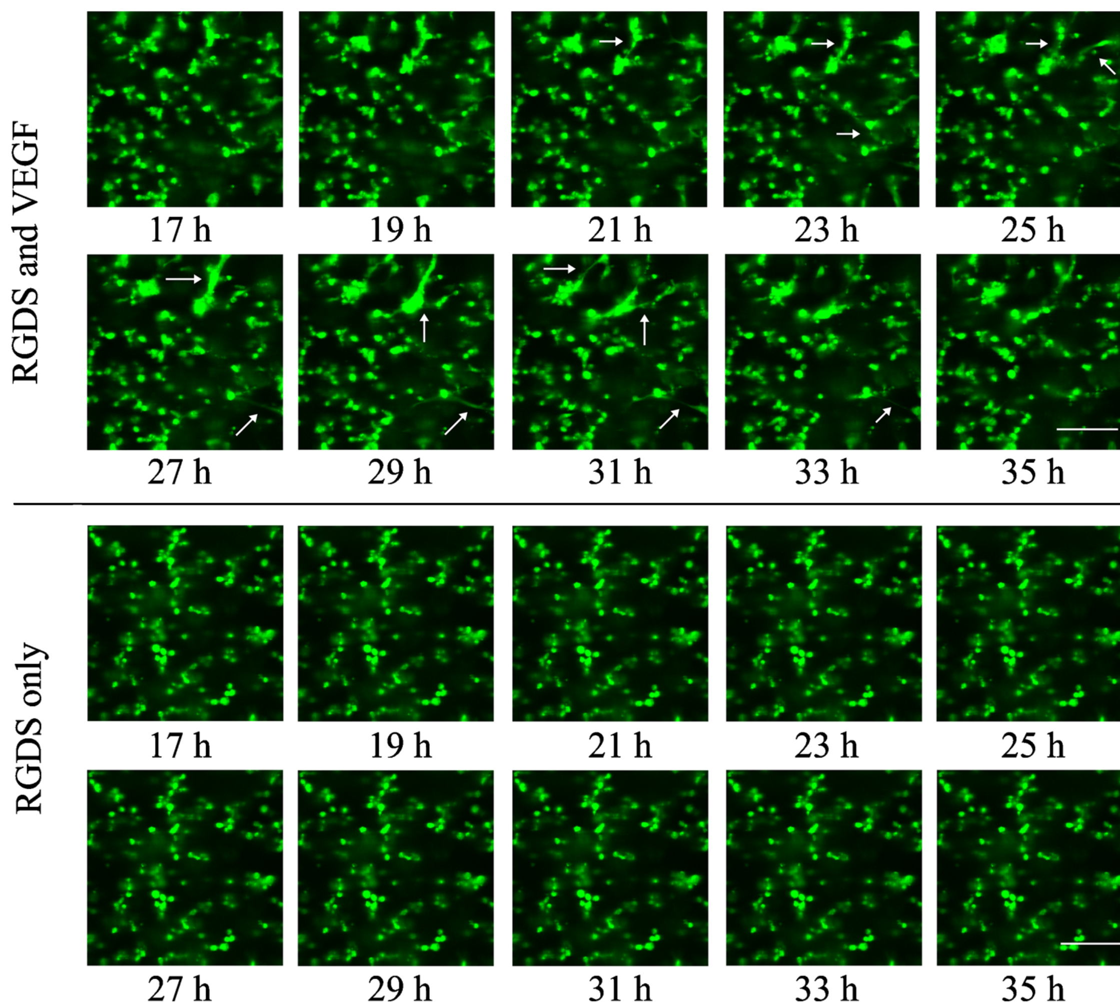


Figure 5. Time series of confocal images illustrating cell behavior in 3D collagenase-degradable PEG hydrogels. Arrows point to cell migration and cell–cell contact formation. (Top) Cellular migration, cell–cell contact formation and branching tube formation inside collagenase-degradable PEGDA hydrogels modified with VEGF and RGDS. (Bottom) Less cellular activity in hydrogels modified with RGDS only. Scale bar = 100 μm. This figure is published in colour in the online edition that can be accessed *via* http://www.brill.nl/jbs

Helm *et al.* studied the requirements for *in vitro* capillary formation in collagen and fibrin gels incorporating matrix-bound VEGF and interstitial flow. After 10 days, blood vessel endothelial cells organized themselves preferably in collagen-fibrin matrices with high fluid permeability and preferred highly compliant matrices, presumably for remodeling and migrating. The researchers noted that because natural scaffolds were used in this study, there were several uncharacterized properties, such as mechanical properties, proteolytic sensitivity, cytokine retention and integrin ligand availability, that could alter the capillary formation processes studied [21]. Complications arise when attempting to understand the results seen in combination natural matrices with uncontrolled signal and integrin ligand densities.

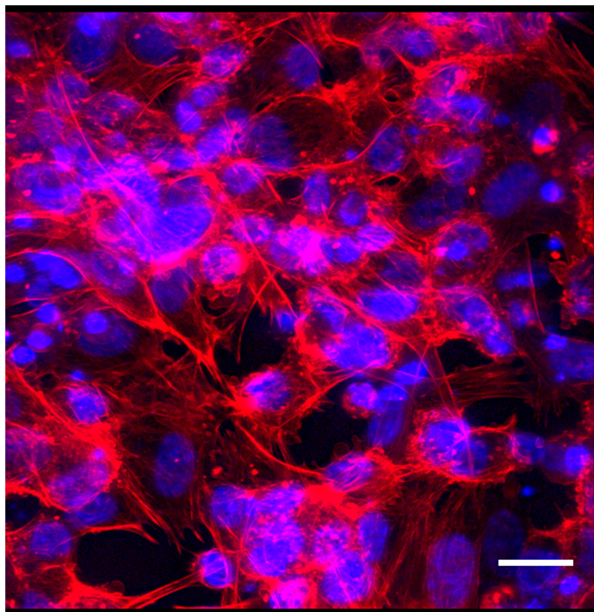


Figure 6. Three-dimensional confocal image of endothelial tube structures formed at 29 h in 3D collagenase-degradable PEG hydrogels modified with VEGF and RGDS. Cellular structures were stained with DAPI (blue) and phalloidin (red) to visualize cell nuclei and actin filaments, respectively. Scale bar = 20 μm. This figure is published in colour in the online edition that can be accessed *via* http://www.brill.nl/jbs

Our results show that covalently immobilized, PEGylated VEGF retains its ability to induce angiogenic behaviors by HUVECs on PEG hydrogels and, thus, may be useful for spatially controlled angiogenesis in engineered tissues. Furthermore, we were able to quantify the tubulogenic process *in vitro*, as a step to assure quality before implantation. Such quality assurance is vital when implanting tissue into compromised patients whose bodies may not be able to vascularize implanted tissues at expected rates.

The time-course of tubulogenesis on 2D PEG hydrogels differs from those studies using natural matrix materials, where tubulogenesis was reported between 5–10 days. We hypothesize that the main reasons for the differences are due to the microenvironment presented to the cells. Matrigel, a largely uncharacterized reconstituted basement membrane material, and fibrin/collagen gels present numerous integrin ligands and sequester many growth factors and other extracellular matrix molecules which can interact with cells. Additionally, these matrices are much softer than the PEGDA hydrogels used in this study, and material stiffness can play a major role in the kinetics of tubulogenesis [21]. The materials used in the present studies were specifically designed to limit integrin and receptor interactions to intentionally immobilized peptides and proteins. Thus, the results observed in our studies are due solely to the scaffold-bound factors and soluble factors in the controlled media. The low level of tubulogenesis seen on RGDS-only gels cultured in VEGF-free EGM-2 is most likely due to bFGF in the media. The presented data shows that PEGylated, covalently-immobilized VEGF has a greater angiogenic ef-

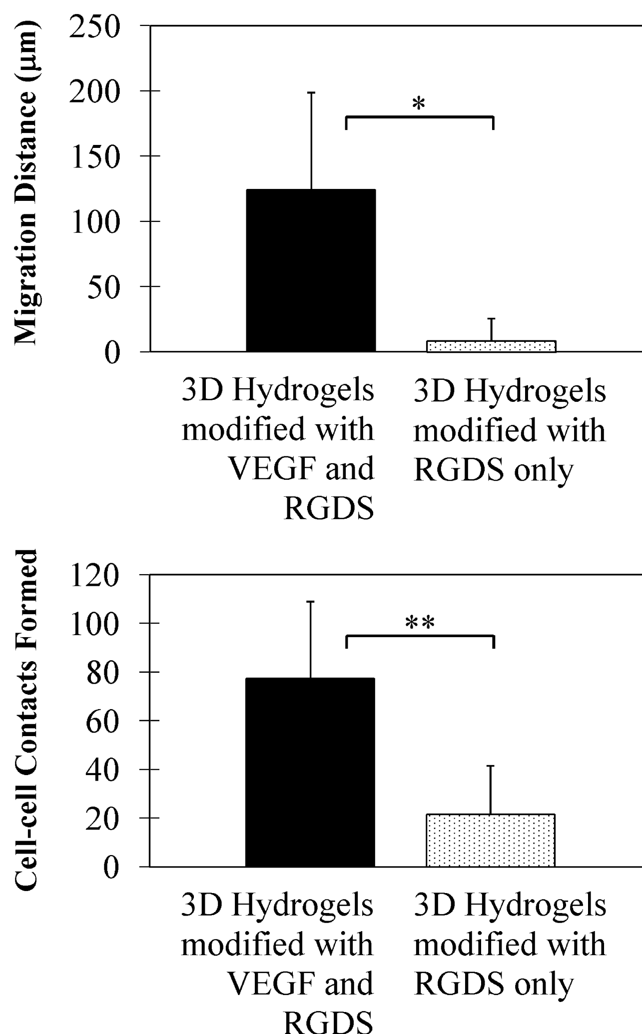


Figure 7. Quantification of angiogenic activity in 3D collagenase-degradable PEG hydrogels shows a significant endothelial cell migration and cell–cell contact response to immobilized VEGF bound within the hydrogel. Error bars show standard deviation (migration $^{*}P < 5.45 \times 10^{-7}$, total contacts $^{**}P < 0.006$).

fect than repeated doses of soluble VEGF and other soluble angiogenic factors, as presented in the media for control groups. An additional advantage of immobilization of VEGF is that it should allow local and controlled angiogenic therapy without unwanted activity elsewhere in the body.

While it took on the order of weeks to observe branching networks of endothelial tubes on the surface of the hydrogels in 2D culture, initial angiogenic activity, including extensive cell migration and cell–cell contact formation, was observed within days for the 3D matrix. We hypothesize that this phenomenon is due to the nature of the matrix. Previous work on RGDS concentrations has shown that a higher level of RGDS in the matrix, which allows more integrin-ligand binding required for extended culture of cells, also reduces cell migration, due to the requirement of cells to detach from the substratum to move forward. In the 2D ex-

periments, PEG-RGDS levels were optimized for long-term attachment, while in the 3D experiments PEG-RGDS levels were optimized for migration [16]. Therefore, while migration and tube formation were observed earlier in 3D matrices, formed tubules on 2D matrices were stable for at least 60 days (data not shown). Furthermore, cells in the 3D matrices received VEGF signaling from all directions, whereas those on the surface of gels received VEGF signaling and integrin attachment ligands only from the basal side of the cell. Thus, the amount of signaling available to cells might have been dissimilar between microenvironments and could have affected the kinetics of tubulogenesis.

Three-dimensional hydrogels allowed extracellular matrix cleavage through the activity of cell-secreted proteases. VEGF has been shown to increase the expression of collagenase by endothelial cells [22]. In matrices with covalently-linked VEGF, cell migration and cell–cell contact formation were significantly higher than in matrices with RGDS only, and cellular spreading and elongation showed similar trends. Because no exogenous collagenase was added to these cultures, the cellular activity suggests that cells in VEGF-matrices produced and secreted more collagenase than those in matrices without VEGF, possibly due to the increased VEGF signaling. Increasing the amount of covalently-bound VEGF in 3D collagenase-degradable hydrogels to 1 nmol/ml accelerated matrix degradation (data not shown), further supporting the specific activity of covalently-bound VEGF on endothelial behavior.

Proteolytically degradable PEG hydrogels provide a controllable system for engineering tissue formation. Degradation kinetics can be modified by varying the concentration of proteolytically degradable peptide in the hydrogel network. Hydrogels consisting of only proteolytically degradable PEG have been studied *in vivo* for 14 days [23], while a combination of degradable/non-degradable hydrogels can be cultured for weeks to months in bioreactor conditions [11]. Zisch *et al.* used biodegradable PEG hydrogels with cleavable, covalently attached $VEGF_{121}$ or $VEGF_{165}$ to induce post-implantation angiogenesis in the scaffold in chorioallantoic membrane and rat models. VEGF was bound to PEG in a manner so that cell-secreted collagenase could cleave and release the protein from the matrix. Their material also contained proteolytically degradable peptide sequences to allow cell invasion. Results showed that these matrices support host tissue generation *in vivo* after 14 days [23]. In the current reported studies, a similar technology was used to further study and enhance the angiogenic potential of PEGDA hydrogels as tissue engineering scaffolds. We show that covalently binding the growth factor directly to the matrix also promotes angiogenic activity in PEG-based hydrogels and that release of growth factor is not needed for this response. The attachment of VEGF to the matrix provides longevity of signaling (shown up to 30 days in this study) and works towards the prevention of undesired angiogenesis in other locations. Micropatterning of the hydrogel materials, achieved using photolithographic techniques, can attain spatial control of capillary formation. In these studies, promotion of microvascularization was achieved *ex vivo*, under non-invasive observation.

5. Conclusions

PEGDA hydrogels have the potential to act as tissue-engineering scaffolds due to their biocompatibility and ability to be tailored for specific applications by incorporating relevant integrin ligands and growth factors to promote desired cell behavior. In this work, PEGDA hydrogels were modified to contain the cell-adhesive peptide RGDS and the angiogenic growth factor VEGF to promote endothelial tubulogenesis, the first step in creating a functional microvasculature in tissue-engineered constructs. Surface immobilization of VEGF significantly enhanced endothelial tubulogenesis, resulting in branching networks of capillary-like tubules on the surface of the hydrogel. Three-dimensional collagenase-degradable PEGDA hydrogels with covalently attached RGDS and VEGF promoted endothelial angiogenic activity by encapsulated HUVEC cells. The incorporation of a collagenase-sensitive peptide sequence within the framework of the hydrogel allowed cells to remodel the matrix during migration and cell–cell contact formation. Covalently immobilized VEGF is a promising avenue for promoting tubulogenesis in engineered tissues. PEG-VEGF covalently attached to the hydrogel retained bioactivity, as evidenced by the continued migratory and contact-forming behavior of cells in VEGF-modified hydrogels. The covalent immobilization of VEGF within the matrix ensures a predicted, local and engineered response. The modification of PEGDA hydrogels with angiogenic signals appears to be a promising method for the generation of microvasculature in tissue-engineered products. The clinical application of this technique may provide pre-vascularized engineered tissues for implantation. Further studies will determine if the incorporation of additional signaling molecules and cell types would provide optimal microvascular formation and stability.

Acknowledgements

This work was supported by an NSF graduate fellowship and NIH.

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