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Biomimetic Poly(ethylene glycol)-Based Hydrogels as Scaffolds for Inducing Endothelial Adhesion and Capillary-like Network Formation

Junmin Zhu*, Ping He, Lin Lin, Derek R. Jones, and Roger E. Marchant*

Department of Biomedical Engineering, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106

Abstract

The extracellular matrix (ECM) is an attractive model for designing synthetic scaffolds with a desirable environment for tissue engineering. Here, we report on the synthesis of ECM-mimetic poly(ethylene glycol) (PEG) hydrogels for inducing endothelial cell (EC) adhesion and capillary-like network formation. A collagen type I-derived peptide, GPQGIAGQ (GIA)-containing PEGDA (GIA-PEGDA) was synthesized with the collagenase-sensitive GIA sequence attached in the middle of the PEGDA chain, which was then copolymerized with RGD capped-PEG monoacrylate (RGD-PEGMA) to form biomimetic hydrogels. The hydrogels degraded in vitro with the rate dependent on the concentration of collagenase, and also supported the adhesion of human umbilical vein ECs (HUVECs). Biomimetic RGD/GIA-PEGDA hydrogels with incorporation of 1% RGD-PEGDA into GIA-PEGDA hydrogels induced capillary-like organization when HUVECs were seeded on the hydrogel surface, while RGD/PEGDA and GIA-PEGDA hydrogels did not. These results indicate that both cell adhesion and biodegradability of scaffolds play important roles in the formation of capillary-like networks.

Keywords

biomimetic hydrogel; poly(ethylene glycol) (PEG); enzyme-sensitive peptide; capillary-like network; tissue engineering; human vein umbilical endothelial cell (HUVEC)

1. INTRODUCTION

Tissue engineering has emerged as a promising method to provide patient-specific tissue substitutes for the replacement or reconstruction of tissues and organs damaged by disease, trauma, or congenital defects.^{1–4} This approach has the advantage to overcome the issues in traditional allogeneic transplantation surgeries, such as donor site morbidity and extended immunosuppression.^{5,6} However, most approaches for engineering new tissues have relied on the host for vascularization, and the current tissue-engineered constructs have been limited to avascular or thin tissues, such as cartilage, skin, or bladder.^{7–11} In these tissues, oxygen and nutrients can diffuse into the implants and sustain cellular viability. However, as the tissue becomes thicker, cells and tissues located 100–200 μm away from nearest capillaries suffer from hypoxia and apoptosis.¹² Thus, vascularization of tissue-engineered constructs remains a great challenge for the development of more complex tissues or organs such as heart, kidney and lung.^{3,13,14}

Blood vessels are developed via two synergistic processes, vasculogenesis and angiogenesis, both being of crucial importance in the formation of vascular networks.^{15–18} Vasculogenesis is the *in situ* assembly of capillaries from undifferentiated endothelial cells (ECs), while angiogenesis is a morphogenic process involving the sprouting of capillaries from pre-existing blood vessels. Vasculogenesis and angiogenesis are important and complex processes involving extensive interplay between cells, matrices and growth factors.^{19,20} The development, maturation and maintenance of the vascular network are necessary for the successful bioengineering of thick tissues or large organs. Several strategies have been explored to enhance vascularization of tissue-engineered constructs,^{21–24} including (i) *in vitro* prevascularization, such as cell culturing^{25–29} and coculturing^{30–33} on or within scaffolds, cell self-assembly,³⁴ and vascularization of spatially-patterned microstructured scaffolds,^{35–39} (ii) direct delivery of growth factors *in vivo*,^{40–43} and (iii) *in vivo* vascularization using cell-seeded scaffolds^{44,45} or direct cell transplantation.^{46,47}

Scaffolds play a crucial role in tissue engineering constructs, including providing support and space for cells, encouraging cell adhesion and growth, and guiding cell function.^{48,49} Various materials have been used as scaffolds for vascularization, including: (i) natural polymers, such as collagen,^{25,33,44} fibrin,⁵⁰ fibrinogen,²⁸ Matrigel,^{51–53} and protein/polysaccharides,⁵⁵ and (ii) synthetic polymers, such as poly(L-lactic acid) (PLLA)/poly(glycolic acid) (PGA)/poly(lactic-glycolic acid) (PLGA),^{55–57} poly(ethylene glycol) (PEG) and PEG derivatives.^{39,58,59} To mimic the native vasculature, microfabrication has been used to design scaffolds with incorporated grooves, microchannels and/or patterned micronetworks to aid in directing cell growth and behavior and act as blood vessel templates for vascularization of bioengineered tissues.^{35–39}

Hydrogels have become an important class of tissue engineering scaffolds, because they can provide a soft tissue-like environment for cell growth and allow diffusion of nutrients and cellular waste through the elastic network.^{49,60} Hydrogel networks can be created by natural, synthetic, or their hybrid polymers. Synthetic hydrogels have advantages over natural hydrogels, such as the ability of photopolymerization, injectable capacity, and adjustable mechanical properties. PEG-based hydrogels have been the primary choice of synthetic hydrogel materials for tissue engineering because they are biocompatible, non-immunogenic, resistant to protein adsorption, and easy for specific modification.⁵⁹ However, synthetic hydrogels usually exhibit no intrinsic biological activity. To incorporate bioactivity, proteins, short peptide sequences and growth factors like VEGF have been used to modify synthetic hydrogels.⁴⁹ Bioactively modified synthetic hydrogel (e.g., PEG and polyacrylamide) have been explored for inducing the formation of microvascular networks both *in vitro* and *in vivo*.^{61–65} Research also has shown that microfabrication of bioactive PEG hydrogels can guide endothelial tubulogenesis³⁹, while co-culturing of endothelial cells with other cells can enhance 3D tubule formation *in vitro*.⁶⁵ However, there was limited research focused on two-dimensional (2D) tube formation on bioactively modified PEG hydrogels.

Here, we report on the synthesis of biomimetic PEG hydrogels for inducing EC adhesion and capillary-like network formation. A collagen type I-derived peptide, GPQGIAGQ (GIA) containing-PEGDA (GIA-PEGDA) was synthesized with the collagenase-sensitive GIA peptide attached in the middle of the PEGDA chain. This was then copolymerized with RGD capped-PEG monoacrylate (RGD-PEGMA) to form extracellular matrix (ECM)-mimetic PEG hydrogels with both collagenase-sensitive degradation and cell-specific adhesion properties. This research seeks to evaluate material parameters and mechanisms that mediate EC adhesion and capillary-like network formation.

2. EXPERIMENTAL

2.1. Materials

All reagents were used without further purification. 9-Fluoromethoxycarbonyl (Fmoc)-protected amino acids, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were purchased from AnaSpec (San Jose, CA). PAL resin (Fmoc-aminomethyl-3,5-dimethoxyphenoxy-valeric acid) with a loading of 0.6 mmol/g, N,N-dimethylformamide (DMF), and N,N-diisopropylethylamine (DIEA) were purchased from Sigma-Aldrich. Acryloyl-PEG-carboxy N-hydroxysuccinimide (Acr-PEG-NHS) was purchased from Laysan Bio (Arab, AL), and its actual Mw was determined by MALDI mass analysis with a maximum peak at 3,700. PEGDA was synthesized according to the method reported previously,⁶⁶ and its actual Mw was determined by MALDI mass analysis with a maximum peak at 6,303. Irgacure 2959 (2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propane) was provided by Ciba (Tarrytown, NY).

2.2. Instrumentation

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was performed on a Bruker Autoflex III MALDI-TOF-MS (Matrix Assisted Laser Desorption/Ionization – Time-Of-Flight Mass Spectrometer) equipped with a standard linear detector and a gridless reflectron detector. Samples were dissolved in 1:1 (v/v) ethanol and water, and mixed with the matrix solution of 2,5-dihydroxybenzoic acid before deposition on the stainless steel sample plate. Mass spectra were typically accumulated from 500 laser shots. Phase contrast photomicrographs of cells were obtained on a Nikon Diaphot 200 Inverted Phase Contrast Microscope with a CCD camera using Metamorph software package.

2.3. Peptide synthesis

Peptides of GPQGIAGQ-Dap (GIA-Dap) and GRGDSP (RGD) were synthesized in a 0.25 mmol scale with PAL resin to produce an amide C-terminus on a 433A Automatic Peptide Synthesizer (Applied Biosystems). Protected diaminopropionic acid (Dap), Fmoc-Dap(Boc)-OH was used to cap the C-terminus of GPQGIAGQ to generate GIA-Dap with two free amine groups on both ends. In general, couplings were performed with a 4-fold excess of Fmoc-amino acid in the presence of HBTU/DIEA/HOBt (1:1:0.5, v/v/v) for 30 min. Fmoc groups were cleaved with 20% (v/v) piperidine in DMF for 10 min. The peptides were cleaved from the resins using the peptide cleavage cocktail of Reagent K containing 85% (v/v) TFA according to the method described previously,⁶⁷ and were further purified by reverse phase-HPLC on a Waters 2690 Alliance system. MALDI-TOF MS was used to confirm their structure.

2.4. Synthesis of GIA-PEGDA and RGD-PEGMA

GIA-PEGDA was synthesized by reacting GIADap with Acr-PEG-NHS. In brief, GIA-Dap was dissolved in 50 mM of sodium bicarbonate buffer (pH 8.2). Acr-PEG-NHS with two-fold molar amount as the GIA peptide, was dissolved in 50 mM of sodium bicarbonate buffer (pH 8.2), and added dropwise to the aqueous peptide solution. The mixture was stirred at room temperature for 24 h, and then dialyzed against water with membranes of Mw Cutoff 5,000 for 48 h. The final product was obtained by lyophilization for 48 h. RGD-PEGMA was synthesized by the same method using an equal molar ratio of GRGDSP and Acr-PEG-NHS, and the final product was dialyzed against water with membranes of Mw Cutoff 3,500 for 48 h. The final structures of GIA-PEGDA and RGD-PEGDA were confirmed by MALDI-TOF mass analysis.

2.5. Fabrication of hydrogels and measurement of mass swelling ratio (Q_m)

Hydrogel solution containing 10–20% (w/v) of total PEG macromers (including GIA-PEGDA, RGD-PEGMA and PEGDA) and 0.1% (w/v) of Irgacure 2959 in PBS was added to each well of a 6-well stainless steel mold. The plate was placed under a UV lamp (365 nm, 5–10 mW/cm²) for 10 min irradiation. Hydrogel disks (8 mm in diameter, 1 mm in thickness) were obtained by lifting the gel mold top plate and transferring the hydrogel disks to glass vials, for measuring mass swelling ratio (Q_m) gravimetrically. Briefly, PBS (4 ml) was added to the hydrogel disks in the glass vials and incubated in a 37°C shaker (60–80 rpm) for 48 h. The swollen hydrogels were then transferred out to a tray, with excess fluid removed by tissue paper, and weighed to obtain the wet weight of the swollen hydrogel (W_{wet}). The swollen hydrogel disks were freeze-dried to obtain the dry weight of the hydrogel (W_{dry}). The Q_m of hydrogels was calculated by using the equation: $Q_m = (W_{wet} - W_{dry})/W_{dry}$.

2.6. Cell culturing on hydrogels

Hydrogel solution with 10% (w/v) of total PEG macromers and 0.1% (w/v) of Irgacure 2959 in PBS was sterilized by filtering through a 0.2- μ m nylon syringe filter, and then added to each well of a sterilized 6-well stainless steel mold to make hydrogel disks using the method described previously. Hydrogel disks were transferred to a 24-well culture plate to swell overnight, before cell seeding. Human umbilical vein endothelial cells (HUVECs) (passage 3–6, Lonza) were cultured with endothelial growth medium (EGM) (EGM Bullet Kit, Lonza), prepared according to the manufacturer instructions, with endothelial basal medium (EBM) supplemented with 2% FBS, bovine brain extract (BBE), human epidermal growth factor (hEGF) and hydrocortisone. Cells were grown to 80–90% confluence, and then trypsinized and resuspended in EBM supplemented with 2% FBS. Silicone O-rings with similar size to the well of the 24-well tissue culture plate were added to sit on the top of hydrogel disks in each well, followed by adding HUVEC suspension (0.5 ml) with seeding density 2.5×10^4 – 1.0×10^5 cells/cm² and incubating at 37°C and 5% CO₂. O-rings were removed 2 h after cell seeding, and cell culture media was replaced with fresh EBM supplemented with 2% FBS. Culture media was changed every 2 days. Cells were imaged on a Nikon Diaphot 200 Inverted Phase Contrast Microscope. Cell coverage area fractions were quantified by ImageJ analysis. ANOVA with Tukey's comparison of means ($\alpha = 0.05$, Minitab 15) was used to assess statistical differences.

3. RESULTS AND DISCUSSION

3.1. Rational design of hydrogel tissue engineering scaffolds

The requirements of scaffolds for tissue engineering include biocompatibility, biodegradability, cell specific adhesion, high porosity and no immunogenic reactions. To promote cellular functions, it is highly desirable that the scaffolds have cell specific adhesion and enzyme-sensitive degradation, which is responsive to signalling molecules and tissue formation. Owing to their design flexibility, PEG hydrogels have been the primary choice for making porous scaffolds with similar characteristics to certain soft tissues such as cartilage. However, the major limitation of PEG hydrogels is the lack of cell-mediated biodegradation and cell specific bioactivities, such as cell adhesion and migration. To overcome this limitation, it is highly desirable to synthesize hydrogel scaffolds that mimic the structure and functions of the natural ECM.

The ECM is a complex network structure that surrounds and supports cells. Cell receptors bind both soluble and tethered signalling cues from the ECM environment, while simultaneously, cells send out signals to construct and degrade their microenvironment for remodelling (Figure 1A). Thus, the natural ECM acts not only as a mechanical scaffold for

the cells, but also a bioactive and dynamic environment that mediates cellular functions. To meet the diverse need in tissue engineering, PEG hydrogels have been modified with the incorporation of bioactive molecules, such as cell adhesive peptides (CAPs), enzyme-sensitive peptides (ESPs) and growth factors (GFs), to mimic one or more ECM biofunctions, such as specific cell adhesion, enzyme-sensitive degradation, and GF-binding. In this manuscript, we report on the copolymerization of a collagenase-sensitive peptide, GPQGIAGQ (GIA) containing-PEGDA (GIA-PEGDA) and RGD capped-PEG monoacrylate (RGD-PEGMA) to form biomimetic hydrogels (Figure 1B) with both collagenase-sensitive degradation and cell-specific adhesion properties for inducing cell adhesion and capillary-like network formation.

3.2. Synthesis of collagenase-sensitive GIA peptide

The proteolytic degradation of the natural ECM is an essential feature of a variety of biological processes, such as cell migration, tissue repair and remodelling. Collagen type I is the most abundant ECM structural protein in mammals, and features a collagenase-sensitive domain with cell-mediated biodegradation to control ECM remodelling and tissue formation. There are two peptide sequences, GPQG↓IAGQ (GIA, from two $\alpha 1$ chains) and GPQG↓LLGA (GII, from $\alpha 2$ chain) (↓ indicating the cleavage site),^{68,69} which are responsible for the proteolytic degradation of collagen by enzymes like collagenase or other matrix metalloproteinases (MMPs) (Figure 2A). The research in this paper focuses on using the GIA sequence for biomimetic modification of PEG hydrogels.

The GIA peptide has only one free amino group which is on the N-terminus of GPQGIAGQ, and thus, is unable to react with two molecules of Acr-PEG-NHS to create a diacrylate-like structure. To provide an additional free amine on the GIA to facilitate conjugating with two molecules of Acr-PEG-NHS to form a GIA-PEGDA macromer, the C-terminus of GIA was capped with diaminopropionic acid (Dap) on a PAL resin by SPPS to generate GPQGIAGQ-Dap (GIA-Dap). The advantage of using Dap instead of lysine (Lys) is that Dap with a shorter alkyl side chain is less hydrophobic than Lys, which benefits the subsequent conjugation reaction with Acr-PEG-NHS. GIA-Dap was cleaved from the resin by Reagent K, and purified by reverse-phase HPLC. The final structure of GIA-Dap (calculated mass 812.0) was confirmed by MALDI mass analysis, with three peaks at m/z = 812.8, 834.2 and 850.5 responding to the ionized molecules of GIA-Dap in the forms of $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$, respectively (Figure 2B).

3.3. Synthesis and characterization of GIA-PEGDA and RGD-PEGMA

GIA-PEGDA was synthesized by conjugating the two free amino groups with Acr-PEG-NHS (Mw 3,700) in sodium bicarbonate buffer (pH 8.2) (Figure 3A). Final products were purified by dialysis against water with Mw cutoff of 5,000, and the final structure was confirmed by MALDI mass analysis. Figure 3B shows a unique distribution with a repeating Mw difference of 44, which corresponds to one PEG repeating unit. The maximum mass peak was at 7,996, indicating the structure of GIA-PEGDA with successful conjugation of one molecule of GIA-Dap (Mw 812) with two molecules of Acr-PEG-NHS (Mw 3,700) with elimination of two molecules of N-hydroxyl succinimide (NHS, Mw 115).

To incorporate the cell-adhesive property into hydrogels, we also synthesized RGD-PEGMA by conjugating an RGD peptide, GRGDSP with Acr-PEG-NHS using the above conjugation method (Figure 3A). The final structure was confirmed by MALDI mass analysis with a maximum peak at 4,178 (Figure 3C), which confirms successful conjugation of one molecule of GRGDSP (Mw 581) with one molecule of Acr-PEG-NHS (Mw 3,700) with elimination of one molecule of NHS (Mw 115).

3.4. Hydrogel preparation and characterization

Hydrogels with 10–20% (w/v) of macromers were fabricated in the form of thin disks (8 mm in diameter, 1 mm in thickness) by photopolymerization at 365 nm for 10 min. Irgacure 2959 was used as a free radical photoinitiator, since it is highly efficient, water-soluble, and low cellular toxicity.^{70,71} GIA-PEGDA can form crosslinked three-dimensional (3D) hydrogel networks by free radical polymerization due to its similar diacrylate structure to PEGDA. The hydrogel swelling properties and cross-linking density are particularly important from a tissue-engineering perspective, since they impact transport and cell viability, and influence cell behavior. The mass swelling ratio (Q_m) and other physical parameters are listed in Table 1. The number-average molecular weight between cross-links (M_c) and average mesh size (ξ) were calculated according to the methods described by Peppas and Hubbell.^{72,73}

Under the same processing conditions, hydrogels made from 10% (w/v) GIA-PEGDA exhibited higher swelling ratio with $Q_m = 45.1$ than the control hydrogels prepared from 10% (w/v) PEGDA ($Q_m = 17.1$). As a result, 10% (w/v) GIA-PEGDA hydrogels have a lower cross-linking density with a higher M_c (3,518 g/mol) and greater mesh size ($\xi = 141.2$ Å), compared with 10% (w/v) PEGDA hydrogels ($M_c = 1,842$ g/mol, $\xi = 74.9$ Å). When the concentration of GIA-PEGDA was increased from 10% to 20% (w/v), GIA-PEGDA hydrogels exhibited lower swelling ratio ($Q_m = 16.3$), which indicates the 20% (w/v) GIA-PEGDA hydrogels have higher crosslinking density with lower M_c (2,662 g/mol) and mesh size ($\xi = 88.0$ Å). This trend is similar to results for the control PEGDA hydrogels with concentration increases from 10% to 20% (w/v) (Table 1). The results demonstrate that hydrogels prepared from photopolymerization of GIA-PEGDA were well-constructed with adjustable physical properties.

3.5. Enzymatic degradation of GIA-PEGDA Hydrogels *in vitro*

The collagenase-sensitive sequence of GPQGIAGQ (GIA) is derived from the collagenase-sensitive domain of collagen type I. To test the collagenase-sensitive degradation of GIA-modified PEG hydrogels, 20% (w/v) GIA-PEGDA hydrogels were incubated in the presence of collagenase with concentrations from 1–20 µg/ml at 37°C (Figure 4). In the absence of collagenase, GIA-PEGDA hydrogels showed no degradation, as indicated by no mass gain or loss over 80 h. However, in the presence of collagenase, GIA-PEGDA hydrogels gained mass during the early phase of enzymatic degradation. This is attributed to a decrease in crosslinking density with increased water swelling of the hydrogels. When the degradation passes a critical time point, the crosslinking density becomes too low to maintain the hydrogel network and hold the water. As a result, the hydrogel network collapses and fully degrades. Figure 4 shows that GIA-PEGDA hydrogels were degraded completely by 85 h with 1 µg/ml collagenase, and by 6 h with 20 µg/ml collagenase. This indicates the degradation rate of GIA-PEGDA hydrogels was dependent on the concentration of collagenase.

3.6. Effect of RGD on cell adhesion to PEG hydrogels

Conventional PEG hydrogels without bioactive modification typically exhibit no intrinsic biological activity due to the bioinert property of PEG. To induce cell specific adhesion, 1% (w/v) RGD-PEGMA was copolymerized with 9% (w/v) PEGDA, to make RGD-modified PEGDA (RGD/PEGDA) hydrogels. To evaluate cell adhesion on the resultant materials, HUVECs (5×10^4 cells/cm²) were seeded on the hydrogel surfaces. Phase contrast images show that HUVECs seeded on PEGDA hydrogels assumed a rounded morphology with no evidence of spreading at both 4 h and 24 h time points (Figure 5A & 5B), suggesting that HUVECs have only weak, non-specific interactions with PEGDA hydrogels. However, when 1% (w/v) RGD-PEGMA was incorporated PEGDA hydrogels (RGD density = 2.5

mM), HUVECs showed higher initial cell attachment after 4 h (Figure 5C) and cell spreading by 24 h (Figure 5D). Quantitative analysis showed that the cell coverage area on RGD/PEGDA hydrogels was more than 30% for both 2 h and 24 h, but less than 5% for the PEGDA hydrogel controls (Figure 5E). In addition, another control of hydrogels from copolymerization of scrambled RGE peptide, GRGESP-capped PEG monoacrylate (RGE-PEGDA) (1%, w/v) and PEGDA (9%, w/v) were used for HUVECE adhesion under the same conditions, and produced similar results to the control of PEGDA hydrogel (10%, w/v) (data not shown). Thus, the enhanced cell adhesion and spreading is attributed to the specific binding of RGD ligands to the integrin receptors on HUVECs.

3.7. Effect of cell seeding density on cell adhesion to PEG hydrogels

Research on 2D tubule formation on ECM-derived hydrogels like Matrigel has shown that the initial cell seeding density and cell adhesion are important for the formation of well-defined capillary-like network.⁷⁴ To evaluate the effect of initial cell seeding on cell attachment on hydrogel surfaces, cells with various seeding densities from 2.5×10^4 to 1.0×10^5 cells/cm² were seeded on 1% (w/v) RGD/PEGDA hydrogels. Phase contrast images (Figures 6A–D) show that when the cell seeding density was increased, more cells were attached on the RGD/PEGDA hydrogel surface by 24 h after cell seeding. Quantitative analysis showed that the cell coverage area increased from 22% to 55% when the cell seeding density was increased from 2.5×10^4 to 1.0×10^5 cells/cm² (Figure 6E). In contrast, HUVECs on the PEGDA hydrogel controls remained in round cell morphology with no spreading, no matter how high the cell density was seeded (data not shown). The results demonstrate that the cell attachment is related significantly to the initial seeding density of HUVECs and to the incorporation of integrin-binding RGD into PEGDA hydrogels, and the incorporation of 1% (w/v) RGD-PEGMA is sufficient to support initial cell adhesion.

3.8. Cell adhesion and tube formation on RGD/GIA-PEGDA hydrogels

Desirable tissue formation requires the cells to express signals to control the biodegradation of synthetic scaffolds like the natural remodelling of the ECM. PEGDA hydrogels have been the primary choice of hydrogel materials for making porous scaffolds. However, they are limited in providing an ideal environment for cells, because the resultant PEG network is not naturally degradable. To address this issue, we synthesized GIA-PEGDA with the GIA peptide attached in the middle of PEGDA chain, and copolymerized it with RGD-PEGMA to make RGD-modified GIA-PEGDA (RGD/GIA-PEGDA) by photopolymerization. To evaluate the cellular response to biomimetically modified hydrogels, we seeded HUVECs (5.0×10^4 cells/cm²) on hydrogels prepared from the copolymerization of 1% (w/v) RGD-PEGMA and 9% (w/v) GIA-PEGDA and used 10% (w/v) GIA-PEGDA hydrogels as control.

Phase contrast images show that HUVECs seeded on GIA-PEGDA hydrogels were round and had no spreading at both 4 h and 24 h time points (Figure 7A & 7B), suggesting that GIA-PEGDA hydrogels were unable to support the initial adhesion of HUVECs due to no cell adhesion ligands on the hydrogel surfaces. This result is similar to the HUVEC seeding on PEGDA hydrogels. When 1% (w/v) RGD-PEGMA was incorporated GIA-PEGDA hydrogels (RGD density = 2.5 mM), HUVECs showed initial cell attachment and spreading at 4 h (Figure 7C), cell migration, elongation and initial reorganization at 12 h (Figure 7D), and capillary-like network formation (Figure 7E). Zooming in the boxed area in Figure 7E shows a multicellular structure for the resultant microvascular tubes and branches formed on the RGD/GIA-PEGDA hydrogels (Figure 7F).

Quantitative analysis shows that the cell coverage area on 1% (w/v) RGD/GIA-PEGDA hydrogels is more than 30% for both 4 h and 24 h, while the cell coverage area on the

control of PEGDA hydrogels is less than 3% (Figure 8). It indicates that the RGD peptide plays an important role in inducing cell initial adhesion and spreading at 4 h due to the specific binding of RGD ligands on RGD/GIA-PEGDA hydrogels to the integrin receptors on HUVECs. Compared with the RGD/PEGDA hydrogels (Figure 5D) without tube-like structure formation, the cell-adhesive and collagenase-sensitive RGD/GIA-PEGDA hydrogels can induce capillary-like network formation, which demonstrates that the biodegradability of hydrogel scaffolds plays an important role. The inclusion of the degradable GIA peptide allows the cells to migrate and subsequently begin to organize and form microvascular networks.

The main technical challenge in the study of vasculogenesis and angiogenesis is the selection of an appropriate characterization method. Despite the increasing numbers of both *in vitro* and *in vivo* models for evaluating capillary-like networks, the *in vitro* model of 2D tubule formation on the top of ECM-derived gel matrices (e.g., collagen, fibrin or Matrigel) has been a robust, rapid and reproducible assay with reliable readouts, automated computer analysis and multi-parameter assessment.^{74,75} Thus, this 2D tubule assay was used in this research for studying the cellular response and morphological differentiation of HUVECs on the ECM-mimetic PEG hydrogels. In addition, this 2D *in vitro* model can be used to evaluate the effects of substrate stiffness and adhesivity on tubule formation,^{64,65} and some research also has shown the presence of lumen of the tubules formed with Matrigel by electronic microscopy.^{74–77} Figure 7F shows that HUVECs can form tubules with a multicellular structure on 1% RGD/GIA-PEGDA hydrogels; however, it needs more future work to elucidate the effects of hydrogel mechanics and ligand (RGD) binding ability on tubule formation and analyze whether the tubules possess a lumen structure.

4. CONCLUSIONS

Bioactive PEG hydrogels were prepared by photo-copolymerization of GIA-PEGDA and RGD-PEGMA to mimic the cell adhesion and enzymatic degradation properties of the natural ECM. GPQGIAGQ (GIA), a collagenase-sensitive sequence derived the $\alpha 1$ chain of collagen type I was synthesized by SPPS with C-terminus capped with Dap to provide two free amino groups for conjugating with Acr-PEG-NHS to generate GIA-PEGDA. GIA-PEGDA can copolymerize with RGD-PEGMA to form biomimetic hydrogels with both collagenase-sensitive degradation and cell-specific adhesion properties. The hydrogels degraded *in vitro* with the rate dependent on the concentration of collagenase, and supported the initial adhesion of HUVECs. Biomimetic RGD/GIA-PEGDA hydrogels incorporating 1% (w/v) RGD-PEGMA induced capillary-like network formation 24 h after seeding HUVECs on the hydrogel surface, while the controls, GIA-PEGDA and RGD/PEGDA hydrogels did not. The results indicate that both cell adhesion and biodegradability of hydrogel scaffolds play important roles in the capillary-like network formation.

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Figure 1.

(A) Schematic of the natural ECM structure and its interaction with cells. FN, fibronectin; LN, laminin; HA, hyaluronic acid; GAG, glycosaminoglycan; PG, proteoglycan. (B) Model of ECM-mimetic PEG hydrogel with incorporation of enzyme-sensitive peptides (ESPs) and cell adhesive peptides (CAPs).

Figure 2.

(A) Collagenase-sensitive sequences, GPQGIAGQ (GIA) and GPQGIALLGA (GII) in the collagenase-sensitive domain of collagen type I (↓ indicating the cleavage site). (B) MALDI mass spectrum of GPQGIAGQ-Dap (GIA-Dap) prepared by SPPS.

Figure 3.

(A) Synthesis of GIA-PEGDA and RGD-PEGMA macromers. (B) MALDI mass spectra of GIA-PEGDA with a maximum peak at 7,996. (C) MALDI mass spectrum of RGD-PEGMA with a maximum peak at 4,178.

Figure 4. Degradation of GIA-PEGDA (20%, w/v) hydrogels in the presence of collagenase in PBS at 37°C.

Figure 5. Phase contrast images of HUVECs (5×10^4 cells/cm²) seeded on PEG hydrogels. (A, B) PEGDA hydrogels for 4 and 24 h, respectively. (C, D) 1% (w/v) RGD/PEGDA hydrogels for 4 and 24 h, respectively (Scale bar = 100 μm). (E) Quantitative comparison of cell coverage area on PEGDA and 1% (w/v) RGD/PEGDA hydrogels.

Figure 6. Phase contrast images of HUVECs 24 h after seeded on 1% (w/v) RGD/PEGDA hydrogels with various seeding densities. (A) 2.5×10^4 cells/cm²; (B) 5.0×10^4 cells/cm²; (C) 7.5×10^4 cells/cm²; (D) 1.0×10^5 cells/cm² (Scale bar = 100 μm). (E) Quantitative comparison of cell coverage area on the hydrogel surface.

Figure 7. Phase contrast images of HUVECs seeded on collagenase-sensitive hydrogels. (A, B) GIA-PEGDA hydrogels for 4 and 24 h, respectively. (C, D, E) 1% (w/v) RGD/GIA-PEGDA hydrogels for 4, 12 and 24 h, respectively. (F) Zooming in the boxed area in (E). (Scale bar = 100 μ m).

Figure 8. Quantitative comparison of cell coverage area on GIA-PEGDA and 1% (w/v) RGD/GIA-PEGDA hydrogels.

Table 1

Hydrogel mass swelling ratio and structural parameters

Hydrogel	% (w/v)	Mw (g/mol)	Q_m [b]	Mc (g/mol)	ξ (Å)
GIA-PEGDA	10	7,996	44.3 ± 1.1	3,518	141.2
	20	7,996	16.3 ± 0.5	2,662	88.0
PEGDA	10	6,303	17.1 ± 0.7	1,842	74.9
	20	6,303	12.0 ± 0.2	1,791	64.7

[a] initial molecular weight of macromers.

[b] Q_m ± standard deviation (SD)