

# Delivery of Sphingosine 1-Phosphate from Poly(ethylene glycol) Hydrogels

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While protein growth factors promote therapeutic angiogenesis, delivery of lipid factors such as sphingosine 1-phosphate (S1P) may provide better stabilization of newly formed vessels. We developed a biomaterial for the controlled delivery of S1P, a bioactive lipid released from activated platelets. Multiarm poly(ethylene glycol)–vinyl sulfone was cross-linked with albumin, a lipid-transporting protein, to form hydrogels. The rate of S1P release from the materials followed Fickian kinetics and was dependent upon the presence of lipid carriers in the release solution. Delivery of S1P from RGD-modified hydrogels increased the cell migration speed of endothelial cells growing on the materials. The materials also induced angiogenesis in the chorioallantoic membrane assay. Our data demonstrate that the storage and release of lipid factors provides a new route for the induction of angiogenesis by artificial materials.

## Introduction

Angiogenesis is necessary to restore the blood supply to ischemic tissue and to generate complex engineered tissues. The formation of new blood vessels is a multistep process, occurring primarily at sites of vascular injury in adults, mediated by the temporally controlled release of biological factors.<sup>1</sup> Many of the factors that control angiogenesis are released from activated platelets, including platelet-derived growth factor (PDGF),<sup>2</sup> vascular endothelial growth factor (VEGF),<sup>3</sup> and basic fibroblast growth factor (bFGF),<sup>4,5</sup> as well as lipid factors such as sphingosine 1-phosphate (S1P) and lysophosphatidic acid.<sup>6,7</sup> During angiogenesis, endothelial cells are initially stimulated to weaken cell–cell interactions, which increases vascular permeability.<sup>8,9</sup> Endothelial cells then proliferate and migrate through the subendothelial matrix, forming sprouts from the wall of the original vessel.<sup>10</sup> These sprouts develop into tube structures<sup>11</sup> that are stabilized by the recruitment of mural cells to the outer surface of the tube of endothelial cells.<sup>6</sup>

Although delivery of bFGF and VEGF to tissues can promote angiogenesis,<sup>12–14</sup> the newly formed vessels may regress over time.<sup>15,16</sup> S1P has been reported to be a complete angiogenic factor,<sup>17–19</sup> regulating early stages of angiogenesis by promoting endothelial cell migration and proliferation<sup>20,21</sup> and later stages by stimulating endothelial cell entubulation<sup>22</sup> and directing the stabilization of newly formed vessels.<sup>23,24</sup> Knockout of the major endothelial cell S1P receptor, S1P<sub>1</sub>, is lethal in mice at E12.5–14.5 because of incomplete vessel maturation, implying a role for S1P in recruiting mural cells, either directly or indirectly.<sup>25</sup> The ability of S1P to stimulate multiple steps in angiogenesis,<sup>17,19</sup> including the stabilization and maturation of newly formed vessels,<sup>2,24,25</sup> suggests that delivery of S1P from materials may provide a more effective route for the formation of a mature vasculature.

Because of the low solubility of S1P in aqueous solutions, its transport in the blood is facilitated by lipid carriers.<sup>26,27</sup> The

majority of S1P in the blood is bound to albumin and high-density lipoproteins (HDL) via affinity interactions.<sup>28</sup> These reversible interactions could be used as a means of controlling S1P release from artificial materials. However, previous attempts to use the binding properties of albumin to achieve controlled delivery led to the complete release of theophylline, acetaminophen, and hydrocortisone in 9 h.<sup>29</sup>

Poly(ethylene glycol) (PEG) materials display a resistance to protein adsorption<sup>30</sup> and versatility in the incorporation of biological factors.<sup>31,32</sup> Conversion of the endgroups on PEG to Michael-type acceptors allows the addition of peptides or other biological factors to the materials via conjugate addition reactions.<sup>32</sup> Vinyl sulfone endgroups provide high reactivity and a high specificity toward thiols at neutral pH, without a competing hydrolysis reaction. Additionally, reaction of vinyl sulfone with amines in a slightly basic environment is feasible, allowing for reaction of PEG with lysine groups in proteins.<sup>33,34</sup>

By cross-linking PEG–vinyl sulfone with albumin, we have developed a new biomaterial for the delivery of the potent angiogenic lipid S1P. Albumin within these hydrogels sequesters S1P and controls its release. Here, we demonstrate the controlled delivery of S1P from PEG hydrogels, the enhancement of endothelial cell migration on the materials *in vitro*, and the induction of angiogenesis in the chorioallantoic membrane (CAM) assay.

## Methods

**PEG Synthesis.** PEG–octavinyl sulfone (PEG–OVS) was synthesized from eight-armed PEG–OH (mol wt 10 000, Shearwater Polymers, Huntsville, AL) in four steps using a modification of the methods described by Morpurgo et al.<sup>34</sup> In the first step, PEG–mesylate was formed by the reaction of mesyl chloride with PEG under nitrogen. PEG (25 g) dissolved in 300 mL toluene was dried by azeotropic distillation until the final volume was 150 mL. After cooling, 50 mL of dichloromethane (DCM) was added to prevent precipitation of PEG. The solution was cooled on ice for 15 min, and 5.58 mL triethylamine (TEA, 2 equiv) was added. Next, 3.10 mL methane sulfonyl chloride (2 equiv) was added dropwise and allowed to react for 20 h. The TEA

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salt was filtered out, and the total volume was reduced to approximately 20 mL by rotary evaporation. PEG was precipitated by addition of ice-cold diethyl ether, recovered by filtration, and dried under vacuum:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 3.13 ppm (s, 3H,  $-\text{SO}_2\text{CH}_3$ ), 3.61 ppm (PEG backbone), 4.35 ppm (t, 2H,  $-\text{CH}_2\text{OSO}_2-$ ). In the second step, PEG-mesylate was reacted with  $\beta$ -mercaptoethanol to form PEG-hydroxyethyl sulfide. The PEG-mesylate (15.5 g) was dissolved in 150 mL sodium borate buffer (50 mM, pH 8), 3.5 mL of 14.3 M  $\beta$ -mercaptoethanol (4 equiv) was added, and the solution was allowed to react at reflux under nitrogen for 3 h. The PEG was extracted twice with DCM, and the volume was reduced to approximately 20 mL by rotary evaporation. PEG was precipitated by addition of ice-cold diethyl ether, recovered by filtration, and dried under vacuum:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 2.72 ppm (t, 2H,  $-\text{CH}_2\text{S}-$ ), 2.75 ppm (t, 2H,  $-\text{SCH}_2-$ ), 3.61 ppm (PEG backbone). Next, the sulfide was oxidized using hydrogen peroxide to form PEG-hydroxyethyl sulfone. PEG-hydroxyethyl sulfide (12.79 g) was dissolved in 50 mL distilled water with 1.69 g sodium tungstate (0.5 equiv). The solution was cooled to near 0 °C and reacted with 2.09 mL 30% hydrogen peroxide (2 equiv) overnight on ice. The PEG was extracted twice with DCM. The total volume of DCM was reduced to approximately 20 mL by rotary evaporation. PEG was precipitated by addition of ice-cold diethyl ether, recovered by filtration, and dried under vacuum:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 3.32 ppm (t, 2H,  $-\text{CH}_2\text{SO}_2-$ ), 3.39 ppm (t, 2H,  $-\text{SO}_2\text{CH}_2-$ ), 3.61 ppm (PEG backbone), 3.92 ppm (t, 2H,  $-\text{OCH}_2\text{CH}_2\text{SO}_2-$ ), 4.03 ppm (t, 2H,  $-\text{SO}_2\text{CH}_2\text{CH}_2\text{OH}$ ). In the final step, the hydroxyethyl sulfone was converted to a vinyl sulfone. PEG-hydroxyethyl sulfone (7.97 g) was dried by azeotropic distillation in toluene until the final volume was 150 mL. After cooling, 50 mL DCM was added to prevent precipitation of the PEG. The solution was cooled on ice for 15 min, then 2.68 mL TEA (3 equiv) was added. Next, 0.75 mL mesyl chloride (1.5 equiv) was added dropwise, and the solution was allowed to react for 24 h. Another 2.68 mL TEA (3 equiv) was added, followed by 0.75 mL mesyl chloride (1.5 equiv added dropwise), and the reaction was carried out overnight. The TEA salt was filtered out, and the total volume was reduced to approximately 20 mL by rotary evaporation. PEG was precipitated by addition of ice-cold diethyl ether, recovered by filtration, and dried under vacuum:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 3.61 ppm (PEG backbone), 6.04 ppm (d, 1H,  $=\text{CH}_2$ ), 6.35 ppm (d, 1H,  $=\text{CH}_2$ ), 6.79 ppm (q, 1H,  $-\text{SO}_2\text{CH}=\text{CH}_2$ ). PEG-tetravinyl sulfone (PEG-TVS) was synthesized from four-armed PEG (mol wt 10 000, Shearwater Polymers) using the same protocol with 80% endgroup conversion by NMR. PEG-diamine was synthesized as previously described<sup>32</sup> from poly(ethylene glycol), mol wt 3400 (24 g, Sigma, St. Louis, MO):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 2.85 ppm (t, 4H,  $-\text{CH}_2-\text{NH}_2$ ), 3.65 ppm (PEG backbone). End group conversion was 99.1% as shown by NMR.

**Hydrogel Formation.** Hydrogels were formed by a conjugate addition reaction between PEG-OVS and (1) PEG-diamine, (2) fatty acid-free bovine serum albumin (FAF-BSA), (3) bovine fibrinogen, or (4) dithiothreitol (DTT). DTT was dissolved in phosphate buffered saline (PBS; 137 mM NaCl, 8 mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 0.7 mM  $\text{CaCl}_2$ , 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 0.5 mM  $\text{MgCl}_2$ ; pH 7.4) at 0.3 mg per 10  $\mu\text{L}$  PBS. All other precursors were dissolved in PBS by adding 10 mg solid to 50  $\mu\text{L}$  PBS at pH 8.0 (assuming a volume change due to the dissolved precursors to be 1  $\mu\text{L}$  per mg of solid, the precursors were nominally dissolved at 10 mg/60  $\mu\text{L}$ ). PEG-OVS was then mixed with cross-linking molecules at a 1:1 ratio of vinyl sulfone to amine. For albumin and fibrinogen, this ratio was estimated by analyzing the 3D crystal structures of the proteins (Protein Databank IDs: 1AO6 and 1DEQ, respectively) with the Deep View Swiss-Pdb Viewer using the default accessibility option in the software. This analyzes lysines on the basis of surface area solvent accessibility of the residue, but does not necessarily indicate the availability of the amine for reaction with a vinyl sulfone. For albumin, 36 of the 59 lysines were accessible, while for fibrinogen, 65 of 106 were accessible. Optimal ratios of reactants were then determined empirically for each cross-linker by forming gels with different ratios of each component, seeking to

minimize the amount of gel swelling in PBS after cross-linking. For PEG-OVS/PEG-diamine gels, 50  $\mu\text{L}$  PEG-OVS ( $\sim 8.3$  mg) was mixed with 68  $\mu\text{L}$  PEG-diamine ( $\sim 11.3$  mg). For PEG-OVS/albumin gels, the final mixture consisted of 40  $\mu\text{L}$  PEG-OVS ( $\sim 6.6$  mg) and 60  $\mu\text{L}$  albumin ( $\sim 10.0$  mg). For PEG-OVS/fibrinogen, the final ratio was 20  $\mu\text{L}$  PEG-OVS ( $\sim 3.3$  mg) to 50  $\mu\text{L}$  fibrinogen ( $\sim 8.3$  mg). After mixing the precursors, gels were formed in tissue culture plate wells, or, for thin-layer chromatography, the scrape wound assay or the chorioallantoic membrane assay, between glass microscope slides. For the latter method, the solution was pipetted onto the center of a glass microscope slide made hydrophobic with SigmaCote (Sigma, St. Louis, MO). Another hydrophobic slide was clamped on top, using 0.7-mm-thick Teflon spacers at the edges of the slides. The solution contacted only hydrophobic glass and formed a circular disk. The solutions were cross-linked in a 37 °C humidified incubator for 24 h. The gels were then swollen in PBS overnight.

**PEG Hydrogel Swelling.** PEG hydrogels (50  $\mu\text{L}$ ) were formed using PEG-OVS and PEG-TVS cross-linked with albumin, fibrinogen, PEG-diamine, or DTT. The hydrogels were swollen in PBS for 96 h, and each gel was weighed to determine the swollen volume. Densities used in the calculation of volume fractions were as follows: PBS, 1.017 g/mL; PEG, 1.199 g/mL; albumin, 1.364 g/mL; fibrinogen, 1.379 g/mL; and DTT, 1.000 g/mL.

**PEG Hydrogel Mesh Size Estimated Using Electrophoresis.** A modified SDS-PAGE gel was used to characterize albumin diffusion through PEG hydrogels. A 15% acrylamide gel was formed at the bottom of the casting chamber, with a spacer to form a well on the left side of the gel. After the acrylamide solidified, the PEG-OVS/albumin precursor solution was pipetted into the formed well. The casting stand was placed in a humidified 37 °C incubator for 24 h to cross-link the PEG gel. A 7% acrylamide gel was formed over the polymerized gels, followed by a stacking gel.

**S1P Loading.** PEG hydrogels were loaded with S1P using two methods: "postloading" and "preloading". PEG hydrogels were "post-loaded" with S1P by passive diffusion, incubating the formed gels with 500  $\mu\text{L}$  of 10  $\mu\text{M}$  S1P (5 nmol) in PBS containing 0.01% (w/v) FAF-BSA. Following 24 h of incubation with S1P, gels were washed in PBS twice for 1 h and then once for 24 h. PEG hydrogels were "preloaded" with S1P by reacting a solution of PEG-OVS with a solution containing a cross-linker premixed with 5 nmol S1P. S1P-preloaded gels were washed in PBS twice for 1 h and then once for 24 h.

**Thin-Layer Chromatography (TLC).** After washing in PBS, 150  $\mu\text{L}$  S1P-loaded gels were placed into Eppendorf tubes containing 500  $\mu\text{L}$  of 0.4% FAF-BSA in PBS, which was vortexed for 24 h. A small sample of the PBS (5  $\mu\text{L}$ ) was removed from each Eppendorf tube and loaded onto a silica TLC plate (Whatman, Inc., Clifton, NJ). The plates were developed in butanol/acetic acid/water (3:3:1). For visualization, the TLC plate was sprayed with ninhydrin reagent (0.2 g ninhydrin in 100 mL butanol) and heated.

**[ $^{32}\text{P}$ ]S1P Release.** Radiolabeled S1P was produced using the methods of Olivera et al.<sup>35</sup> Briefly, 10  $\mu\text{L}$  of 10 mM sphingosine (BioMol, Plymouth Meeting, PA) in buffer A (25 mM Tris HCl, pH 7.4, 10% (v/v) glycerol, 0.05% Triton X-100, 1 mM DTT) containing 0.4% BSA was added to 80  $\mu\text{L}$  sphingosine kinase (3.6 mg/mL in buffer A) and 90  $\mu\text{L}$  buffer A. The reaction was started by adding 20  $\mu\text{L}$  [ $^{32}\text{P}$ ]ATP (100  $\mu\text{Ci}$ , 10 mM, MP Biomedicals, Irvine, CA) in 100 mM MgCl. The reaction was incubated overnight at 37 °C and stopped by the addition of 20  $\mu\text{L}$  of 1 M HCl. Lipids were extracted with 800  $\mu\text{L}$  chloroform/methanol/1 M HCl (100:200:1). After mixing, 240  $\mu\text{L}$  chloroform and 240  $\mu\text{L}$  of 2 M KCl were added, and the solution was centrifuged. The aqueous phase was removed, and the organic phase was extracted again by the addition of 400  $\mu\text{L}$  of 1:1 methanol/1 M HCl. The phases were separated by centrifugation. The organic phase was removed, dried under nitrogen flow, and dissolved in PBS containing FAF-BSA. For gels postloaded with S1P after cross-linking, [ $^{32}\text{P}$ ]S1P was dissolved in PBS containing 0.01% FAF-BSA and added

to 50  $\mu$ L PEG hydrogels in a 24-well plate for 24 h. For gels formed from albumin preloaded with S1P, [ $^{32}$ P]S1P was dissolved in 570  $\mu$ L PBS containing 95 mg FAF-BSA. Aliquots were mixed with PEG-OVS and incubated for 24 h to form S1P-containing hydrogels (50  $\mu$ L total volume) on the bottoms of wells in a 24-well plate. The S1P-loaded hydrogels were washed twice in 1 mL PBS for 1 h (to remove unbound S1P) and a third time for 24 h. Next, 1.5 mL of platelet-poor plasma (PPP) or PBS containing 0%, 0.4%, or 4% (w/v) FAF-BSA was added to the gels. The solutions were removed from the wells and replaced with fresh solutions at 1, 3, 6, 12, and 24 h, and the release of [ $^{32}$ P]S1P was quantified by scintillation counting. The release at each time point is reported as a percentage of total S1P in each gel, which was determined by summing the amount of S1P in all wash and release solutions for each gel and the amount of S1P in each gel at the end of the experiment. PPP was obtained from human subjects with informed consent in accordance with Washington University Medical Center guidelines (Human Studies Committee Approval #04-097).

**Cell Culture.** All cell culture reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics, Inc. (Walkersville, MD) and cultured in MCDB 131 medium supplemented with 2% FBS, 10 ng/mL epidermal growth factor, 10  $\mu$ g/mL heparin, 1.0  $\mu$ g/mL hydrocortisone, 1% antibiotic-antimycotic (ABAM, 100 $\times$ ) solution (Invitrogen, Carlsbad, CA), and 6 mg/L bovine brain extract (Clonetics).

**Scrape Wound Assay.** Cells from passage 5–7 were grown to confluence in a 6-well tissue culture plate and then switched to low-serum medium (LSM; 0.1% FBS, 1% ABAM in MCDB 131 medium) with 10 ng/mL VEGF and 0.4% FAF-BSA 12 h prior to the start of the experiment (the VEGF was added to prevent cell detachment associated with low-serum medium at 24 h). A 1000  $\mu$ L pipet tip was used to make a vertical and horizontal scrape wound in each well, crossing near the middle of the well. Each well was washed 3 $\times$  with PBS to remove nonadherent cells. At the start of the experiment, pictures were taken at 4 $\times$  magnification on each arm of the scrape wound, and 20  $\mu$ L PEG gels were then added to the appropriate wells. Pictures were taken again after 24 h, using calipers on the microscope to locate the same locations. Image-Pro Express software was used to define the initial scrape wound area, and the number of cells within the wound area was counted manually.

**Chorioallantoic Membrane (CAM) Assay.** Embryonated chicken eggs were incubated blunt-end-up in a 37  $^{\circ}$ C incubator. At embryonal day 6, the top of the egg was cracked and removed using tweezers to create a 1.5-cm-diameter opening. The inner shell membrane was removed, exposing the CAM. A 20  $\mu$ L PEG hydrogel was then placed onto the CAM. As controls, 50 ng bFGF or 50 ng S1P were spotted onto small pieces of Thermanox coverslips (Nunc), air-dried, and placed onto the CAM. The eggs were sealed with Parafilm and returned to the incubator. After 2 days of incubation, the eggs were reopened, and the degree of angiogenesis was visually assessed.<sup>36</sup>

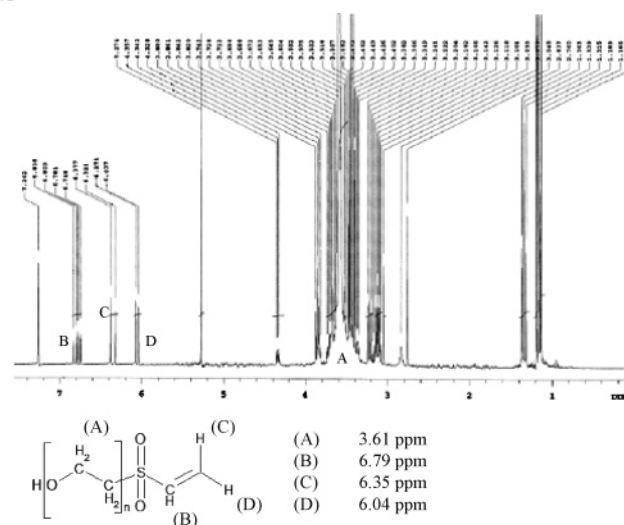
**Sulforhodamine Release.** Sulforhodamine B (500 nmol) was preloaded into gels formed with PEG-OVS cross-linked with albumin, PEG-diamine, or DTT. The release experiment was run using the same protocol as the [ $^{32}$ P]S1P release experiments described above, with detection by visible light spectroscopy at 554 nm.

**Individual Cell Tracking.** PEG-OVS (3.4 mg in 20  $\mu$ L) was reacted for 30 min with acetyl-GCGYGRGDSPG-NH<sub>2</sub> peptide, to attach the peptide to 1/20 of the vinyl sulfone groups (2.78 mM RGD peptide in the final hydrogel). The PEG hydrogels were cross-linked by reacting PEG-OVS with albumin (5.2 mg in 30  $\mu$ L) premixed with S1P (5 nmol) to give a final concentration of 100  $\mu$ M S1P within the cross-linked hydrogel. The resulting solution (50  $\mu$ L) was pipetted into a 24-well plate and incubated at 37  $^{\circ}$ C for 24 h to allow cross-linking. HUVEC from passage 5–7 were seeded onto the gels at a concentration of 1000 cell/cm<sup>2</sup>. After allowing the cells to spread for 6 h in complete growth medium, HEPES-buffered low-serum medium (MCDB 131 with 0.1% FBS, 1% ABAM, 10  $\mu$ M HEPES, pH 7.4) containing 0.4% FAF-BSA

a

8-arm PEG	Endgroup Conversion (%)	Yield (%)
PEG-mesylate	99.1	82.2
PEG-hydroxyethyl sulfide	80.1	88.1
PEG-hydroxyethyl sulfone	84.0	85.1
PEG-vinyl sulfone	74.7	78.5

b



**Figure 1.** Synthesis of PEG-octavinyl sulfone. PEG-octavinyl sulfone was synthesized using a four-step process from eight-armed PEG (mol wt 10 000). (a) The percentage of endgroup conversion for each step in the PEG-octavinyl sulfone synthesis was obtained by comparing the areas of endgroup peaks to the area of the PEG backbone peak. (b) NMR of the PEG-octavinyl sulfone product, with the PEG backbone peak at 3.61 ppm and the vinyl sulfone peaks between 6 and 7 ppm.

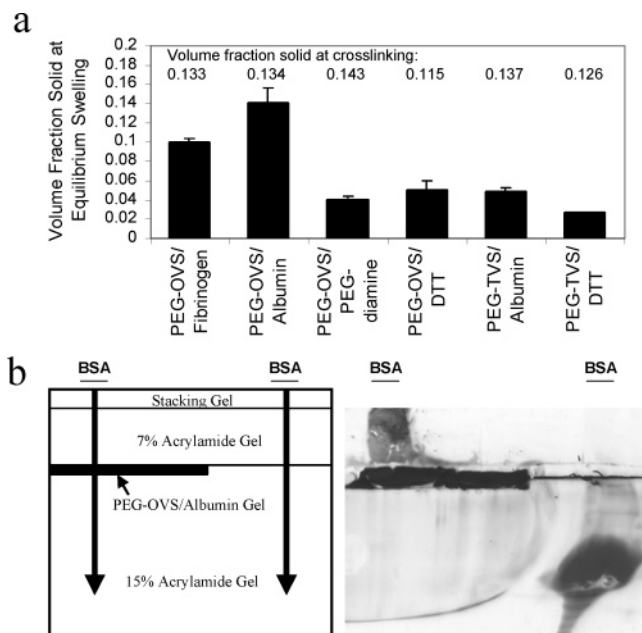
was added to the cells. The cells were tracked using time-lapse microscopy for 12 h, recording images every two minutes. Individual cell migration speeds were analyzed manually using ImageJ to trace the path of each cell over time. The time increment between analysis was increased to 6 min if cells maintained a straight path in the three consecutive images.

## Results

**PEG Gel Formation and Characterization.** We synthesized PEG-OVS using a four-step method, based on the methods of Morpurgo et al.<sup>34</sup> Each step of the synthesis gave about an 80% yield of product, and the extent of endgroup modifications were measured using NMR following each step (Figure 1a). NMR on the final product, PEG-OVS, showed 74.7% endgroup conversion to vinyl sulfone (Figure 1b), with 18.8% of the endgroups present as mesylates. Considering that conversion to the hydroxyethyl sulfide was only 80% complete, it is likely that the mesylates are attached to carbons that are  $\beta$  to ether groups, not sulfones. Thus, the product is only nominally octavinyl sulfone, with typically six vinyl sulfones and one to two mesylate groups per polymer chain. The mesylates may hydrolyze before reaction or may contribute to the cross-linking reaction.

PEG-OVS was cross-linked with molecules containing two or more thiols or amines, forming hydrogels via conjugate addition reactions (i.e., Michael-type addition). At 37  $^{\circ}$ C, PEG-OVS formed a gel with a thiol cross-linker, DTT, in less than 10 min (at pH 7.4) and with amine cross-linkers in approximately 16 h (at pH 8.5). The swelling properties of the



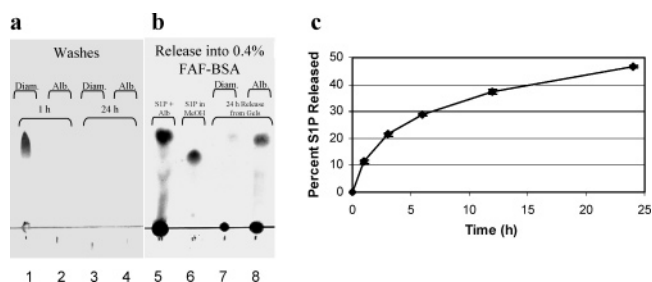


**Figure 2.** Characterization of hydrogel cross-link densities. (a) PEG-octavinyl sulfone (PEG-OVS) or PEG-tetravinyl sulfone (PEG-TVS) was cross-linked with albumin, fibrinogen, PEG-diamine (mol wt 3400), or DTT. The % solid in each swollen gel was calculated after 4 days of swelling in PBS, pH 7.4. The hydrogels were cross-linked at the fraction solids listed above each column. Data represent mean  $\pm$  standard deviation. (b) A 15% SDS-PAGE gel was polymerized, with a spacer on the top left side of the chamber. After the spacer was removed, a thin layer of PEG-OVS/albumin precursor solution was pipetted into the formed well and cross-linked for 24 h. A 7% SDS-PAGE gel was then polymerized to fill the rest of the casting chamber. Bovine serum albumin (BSA; 20  $\mu$ g in 20  $\mu$ L) was loaded on the gel at the indicated locations. BSA did not pass through the PEG gel and accumulated above the hydrogel layer. Note the intense staining of the PEG-OVS/albumin gel with Coomassie Blue and the apparent migration of some proteinaceous material from the PEG gel.

hydrogels were determined for the different cross-linkers. Gels cross-linked with proteins swelled less than gels cross-linked with bifunctional cross-linkers, indicating that at least some of the protein molecules served as elastically active cross-link sites (Figure 2a). Additionally, PEG-OVS/albumin hydrogels swelled less than PEG-OVS/fibrinogen hydrogels. PEG-OVS/albumin gels were exceptional in that they did not appreciably swell or shrink from the volume at which they were cross-linked. This property allowed us to covalently couple these hydrogels to surfaces during cross-linking, leading to gels whose surfaces did not appreciably deform following addition to excess solvent. Typically, gels attached to surfaces during cross-linking will wrinkle or tear while reaching equilibrium swelling volumes.

The mesh size of PEG-OVS/albumin hydrogels was investigated using SDS-PAGE with a PEG-OVS/albumin hydrogel polymerized into the acrylamide gel. Albumin migration through the PEG gel was hindered, resulting in an accumulation of albumin above the PEG layer (Figure 2b). Thus, the mesh size of the hydrogels, which is characterized by the radius of gyration of the polymer chains between elastically active cross-links, is less than or on the order of the radius of gyration of albumin (about 30 Å).<sup>37</sup> The molecular weight markers aprotinin (6.5 kDa) and lysozyme (14.4 kDa) freely passed through the PEG gel (data not shown).

**Loading PEG Hydrogels with S1P.** S1P was loaded into the PEG hydrogels using two techniques, postloading and preloading. For postloading, previously formed PEG hydrogels were placed in solutions containing S1P and 0.01% (w/v) FAF-



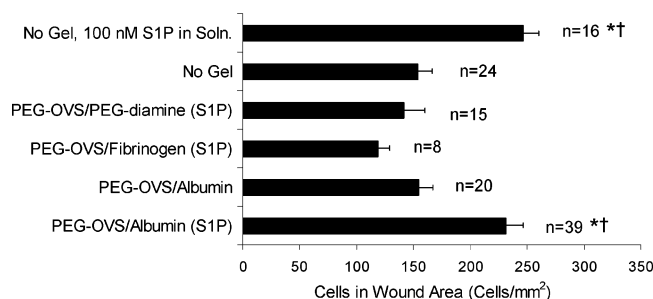
**Figure 3.** S1P release from PEG hydrogels "postloaded" with S1P. PEG hydrogels were formed by reacting PEG-OVS with albumin, fibrinogen, or PEG-diamine. The cross-linked hydrogels were then "postloaded" with S1P by incubation with 5 nmol S1P in 0.01% FAF-BSA for 24 h at 37 °C (all FAF-BSA solutions were in PBS, pH 7.4). This was followed by two washes with PBS for 1 h and a final wash with PBS for 24 h. (a) The contents of the first 1 h PBS wash and the 24 h PBS wash were analyzed by thin-layer chromatography for a PEG-OVS/PEG-diamine gel (Diam.) or a PEG-OVS/albumin gel (Alb.). (b) After the PBS washes, the same gels were then incubated with a solution of 0.4% FAF-BSA in PBS. Lane 5 shows the control consisting of S1P dissolved in 0.4% FAF-BSA, while lane 6 shows S1P dissolved in methanol. Note the difference in  $R_f$  in the presence of FAF-BSA. (c) Release of S1P from PEG-OVS/albumin hydrogels was measured using [<sup>32</sup>P]S1P. In 24 h at 37 °C, the 50  $\mu$ L hydrogels sequestered  $59.1 \pm 3.1\%$  of the S1P from a solution of 5 nmol S1P in 1.5 mL of 0.01% FAF-BSA. During the PBS washes,  $6.9 \pm 1.1\%$  of the loaded S1P was released over 26 h. At time = 0 in the figure, 0.4% FAF-BSA was added to the gels, leading to the release of  $46.6 \pm 0.4\%$  of the S1P in the hydrogel within 24 h. Error bars represent std dev.

BSA. The FAF-BSA in solution was necessary to solubilize the S1P, but the concentration of albumin in the loading solution was much lower than the concentration of albumin in the hydrogel (10% w/v). For preloading, S1P was solubilized directly into the albumin solution used to cross-link the PEG-OVS. After S1P loading, the gels were washed with PBS three times over 26 h to remove weakly bound S1P, unbound S1P, or S1P attached to albumin molecules that were not cross-linked within the gel.

**PEG Gels Postloaded with S1P.** Thin-layer chromatography was used to analyze the release of S1P from postloaded hydrogels. Most of the S1P was washed out of PEG-OVS/PEG-diamine hydrogels in the first 1 h wash with PBS, but PEG-OVS/albumin hydrogels retained the S1P during the washes (Figure 3a). When the hydrogels were placed into a solution containing 0.4% FAF-BSA, a large amount of S1P was released from the PEG-OVS/albumin hydrogels, but apparently little of the S1P remained in the PEG-OVS/PEG-diamine hydrogels to be released (Figure 3b).

We produced <sup>32</sup>P-radiolabeled S1P to quantify loading and release of S1P with postloaded PEG-OVS/albumin hydrogels. [<sup>32</sup>P]S1P (5 nmol in 1.5 mL) was dissolved in PBS containing 0.01% FAF-BSA and incubated with 50  $\mu$ L hydrogels formed in the bottoms of wells of a 24-well tissue culture plate. When PEG-OVS/albumin hydrogels were incubated with 1.5 mL of the 3.33  $\mu$ M S1P solution,  $59.1 \pm 3.1\%$  of the [<sup>32</sup>P]S1P in solution was captured in the gel within 24 h. The gels were then washed thoroughly with PBS for 26 h, with  $6.9 \pm 1.1\%$  of the loaded S1P released into the wash solutions. PBS containing 0.4% FAF-BSA was added to the gels, and controlled release of S1P from the gels was observed, with  $46.6 \pm 0.4\%$  of the remaining S1P released over 24 h (Figure 3c).

An in vitro scrape wound assay<sup>38</sup> was used to demonstrate that S1P released from postloaded gels promoted endothelial cell migration. S1P-loaded PEG gels were placed into tissue culture wells containing scrape-wounded HUVEC in low-serum



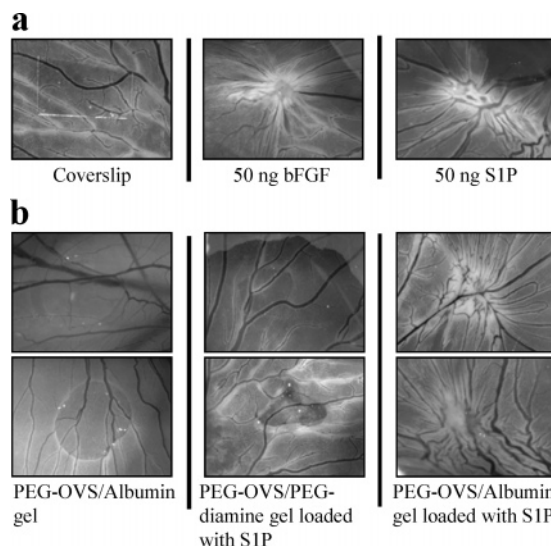
**Figure 4.** A scrape wound assay was used to demonstrate the response of human umbilical vein endothelial cells to S1P released from the hydrogels. PEG-OVS/albumin gels (20  $\mu$ L) postloaded with S1P increased the amount of cell migration into the wound area ( $p = 0.01$  vs medium with no gel,  $p = 0.02$  vs PEG-OVS/albumin gels without S1P loading) to an extent similar to 100 nM S1P in medium ( $p = 0.03$  vs medium with no gel,  $p = 0.02$  vs PEG-OVS/albumin gels without S1P loading). All other experimental conditions did not cause significant differences in cell migration into the wound area vs medium alone. In the row labels, "(S1P)" denotes S1P postloaded into the hydrogels. \*Statistically significant difference vs medium with no gel. †Statistically significant difference vs PEG-OVS/albumin gels without S1P loading. Error bars represent SEM with statistical analysis by ANOVA and postprocessing with the Scheffe test.

medium supplemented with 10 ng/mL VEGF and 0.4% w/v FAF-BSA. The hydrogels were allowed to freely float in the medium. PEG-OVS/albumin gels postloaded with S1P significantly increased the number of endothelial cells that migrated into the scrape wound area by 24 h, compared to wells without gels, gels not loaded with S1P, or PEG-OVS/fibrinogen or PEG-OVS/PEG-diamine hydrogels postloaded with S1P (Figure 4). Considering the results in Figure 3a, the S1P loaded in the fibrinogen and diamine cross-linked hydrogels was likely removed during the washing steps.

A chick CAM assay was used to demonstrate the ability of S1P-releasing hydrogels to induce angiogenesis *in vivo*. PEG hydrogels were placed onto the CAM on embryonic day 6, and the extent of the angiogenic response was determined 2 days later. As controls, bFGF or S1P were spotted on Thermanox coverslips, eliciting a strong angiogenic response (Figure 5a). PEG-OVS/albumin gels not loaded with S1P did not induce an angiogenic response, while PEG-OVS/PEG-diamine gels postloaded with S1P and washed with PBS for 26 h exhibited at most a modest angiogenic response. However, PEG-OVS/albumin hydrogels postloaded with S1P induced a moderate to strong angiogenic response (Figure 5b). Two independent replicates are shown for each condition.

**PEG Gels Preloaded with S1P.** Release of S1P from preloaded PEG-OVS/albumin hydrogels was quantified using [ $^{32}$ P]S1P. The hydrogels were formed in the bottoms of the wells of a 24-well plate. The formed gels were washed with PBS over 26 h and then incubated with solutions containing different concentrations of albumin in PBS. S1P release into PBS alone was limited, while the rate of release increased with higher concentrations of albumin in solution (Figure 6a). Controlled release was observed, with  $33.8 \pm 1.5\%$  of the S1P remaining in the gel after washing released into 4% FAF-BSA in 24 h, and  $26.0 \pm 0.5\%$  of the S1P released into 0.4% FAF-BSA in 24 h. S1P release into human platelet-poor plasma, which contains approximately 4% albumin, displayed release kinetics similar to 0.4% FAF-BSA in PBS. This may be due to competition of S1P released from the hydrogels with lipids already bound to albumin and lipoproteins in plasma.

The effect of gel mesh size on the diffusion of small molecules was tested using a water-soluble dye molecule,

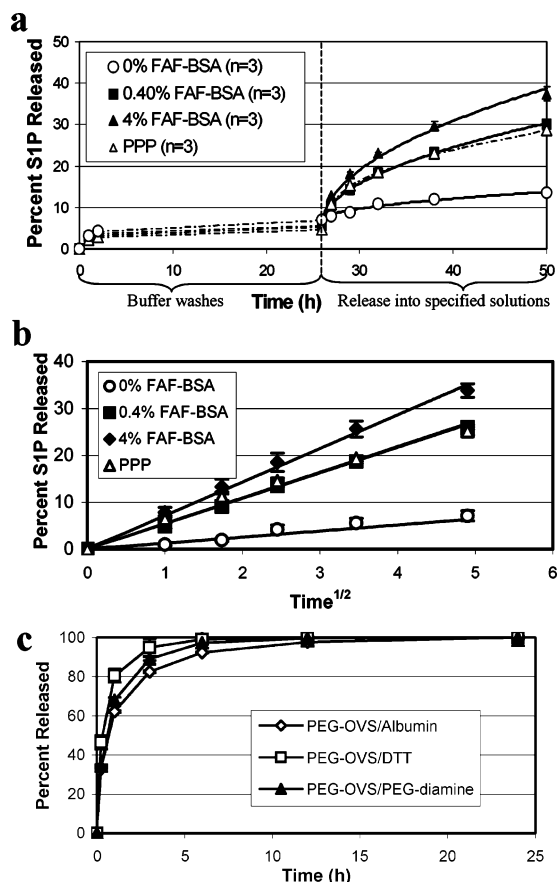


**Figure 5.** Angiogenic response observed in CAM assay. (a) As controls, S1P or bFGF were spotted onto Thermanox coverslips, air-dried, and placed face down onto the chick chorioallantoic membrane (CAM). A strong angiogenic response was elicited by 50 ng bFGF or 50 ng S1P (characterized by the curvature of large vessels toward the point of stimulus). (b) A 20  $\mu$ L PEG-OVS/albumin hydrogel without S1P showed no angiogenic response. A PEG-OVS/PEG-diamine hydrogel loaded with 5 nmol S1P and washed with PBS for 26 h showed little or no angiogenic response. A PEG-OVS/albumin hydrogel loaded with 5 nmol S1P and washed with PBS for 26 h showed a moderate to strong angiogenic response. Results from two separate experiments are shown for each treatment.

sulforhodamine B. The dye was preloaded into PEG-OVS/albumin hydrogels and released into PBS. The molecular weight of sulforhodamine is 580.7, compared to 379.5 for S1P, and the diffusivity of the dye would be expected to be lower than that of S1P. Using the Wilke–Chang correlation (see Supporting Information), we calculated a diffusion coefficient of  $3.42 \times 10^{-6}$  cm $^2$ /s for sulforhodamine and  $4.15 \times 10^{-6}$  cm $^2$ /s for S1P, both at 37  $^{\circ}$ C. Release of sulforhodamine B from the hydrogels was rapid, nearing completion in 6 h (Figure 6c), indicating that the mesh size of the PEG-OVS/albumin gels does not greatly affect the release of small molecules.

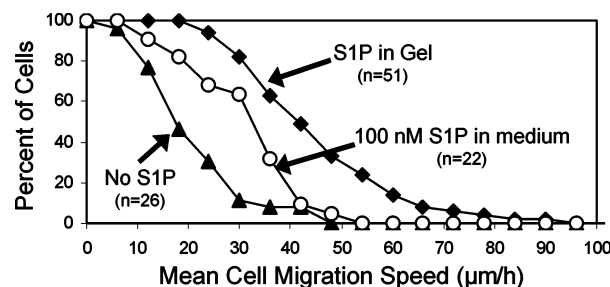
We used time-lapse microscopy to quantify endothelial cell migration speeds on PEG-OVS/albumin hydrogels containing RGD peptide and preloaded with S1P. RGD peptide was added to the PEG hydrogels to promote endothelial cell adhesion and spreading on the materials. After endothelial cells were seeded on the gels, the gels were incubated with complete medium for 6 h containing 2% serum (with a total albumin concentration of about 0.08%). Over the next 12 h, cell migration rates were measured in low-serum medium containing 0.4% FAF-BSA. On the basis of release between 32 and 44 h in Figure 6a, the S1P concentration in the low-serum medium increased from less than 1 nM (low-serum medium contained 0.1% FBS, and FBS contains 142 nM S1P $^{28}$ ) to at least 303 nM by the end of the migration experiment. Cell migration speeds were measured for HUVEC on 50  $\mu$ L PEG/albumin/RGD hydrogels formed on the bottoms of wells of a 24-well tissue culture plate. Delivery of S1P from the hydrogels increased the mean migration speeds of HUVEC from  $19.2 \pm 10.1$   $\mu$ m/h to  $43.8 \pm 15.7$   $\mu$ m/h (Figure 7). The addition of culture medium containing 100 nM S1P increased the mean migration speed to only  $30.2 \pm 11.8$   $\mu$ m/h.

**Analysis of S1P Release Data.** An equilibrium binding constant for the interaction between S1P and albumin has not been reported. Additionally, reported values for equilibrium binding constants for interactions between lipids and proteins



**Figure 6.** S1P release from PEG gels cross-linked with albumin "preloaded" with S1P. S1P was dissolved in PBS containing 17.5% FAF-BSA (1 nmol S1P per mg of albumin). PEG hydrogels (50  $\mu$ L) were formed in the wells of a 24-well plate by adding PEG-OVS to the albumin/S1P solution, with a total of 5 nmol S1P present in each gel. (a) The rate of S1P release was measured using [<sup>32</sup>P]S1P. Approximately 5% of the S1P in the hydrogel was released into the PBS washes over 26 h. The rate of S1P release increased with increasing concentrations of FAF-BSA in the release solution. Release of S1P into human platelet-poor plasma was similar to release into PBS containing 0.4% FAF-BSA. Dashed lines connect data points; solid lines show least-squares fits to a solution of the unsteady diffusion equation (see text). A single parameter was adjusted in the model, an effective diffusion coefficient. Error bars represent standard deviations. (b) The S1P release data was plotted as a function of (time)<sup>1/2</sup> to determine the fit of the data to the short-time Fickian release equation (see text). (c) The effect of gel cross-link density on the release of small molecules was minor compared to the effect of specific binding to albumin. Release of sulforhodamine B, a water-soluble dye molecule 50% larger than S1P, was measured from 50  $\mu$ L PEG-OVS/albumin gels. The dye (500 nmol) was incubated with albumin prior to cross-linking. Release of sulforhodamine B into PBS was nearly complete in 6 h.

often vary by an order of magnitude.<sup>39</sup> The albumin concentration in each PEG-OVS/albumin gel was 1.51 mM (76 nmol in 50  $\mu$ L), and the initial S1P concentration in the preloaded gels was 100  $\mu$ M (5 nmol). The concentration of albumin in the 0.4% FAF-BSA release solution was 60.2  $\mu$ M (75.8 nmol in 1.5 mL). Assuming equilibrium dissociation constants ranging from 1 nM to 10  $\mu$ M, we determined that S1P release from the gels did not reach equilibrium at any of the experimental timepoints in 0.4% FAF-BSA, 4% FAF-BSA, or PPP (see Supporting Information). This indicated that S1P release was controlled by the rate of diffusion of S1P within the gel or the rate of diffusion of dissolved albumin into the gel, and not by the saturation of albumin in the release solutions with S1P.



**Figure 7.** Endothelial cell migration on PEG gels cross-linked with albumin "preloaded" with S1P. S1P was dissolved into PBS containing 17.5% FAF-BSA (1 nmol S1P per mg of albumin). PEG hydrogels (50  $\mu$ L) were formed in the wells of a 24-well plate by reacting PEG-OVS with the albumin/S1P solution, with 5 nmol S1P present in each gel. RGD peptide was also added to the gels to promote cell adhesion and spreading. The migration speed of individual HUVEC seeded on PEG/albumin hydrogels containing RGD peptide was measured in the presence or absence of S1P released from the gel. Delivery of S1P from the hydrogel increased the mean migration speed of the HUVEC from  $19.2 \pm 10.1$   $\mu$ m/h to  $43.8 \pm 15.7$   $\mu$ m/h, while addition of 100 nM S1P to the medium above HUVEC growing on the PEG hydrogel increased the mean migration speed to  $30.2 \pm 11.8$   $\mu$ m/h ( $p = 3 \times 10^{-10}$ , gel + S1P vs gel w/o S1P;  $p = 0.0008$ , gel + S1P vs 100 nM S1P;  $p = 0.02$ , 100 nM S1P vs gel w/o S1P). Statistical analysis was by ANOVA with postprocessing with the Scheffe test.

**Table 1.** Effective Diffusion Coefficients for S1P Release from PEG-OVS/Albumin Hydrogels<sup>a</sup>

	0% FAF-BSA	0.4% FAF-BSA	4% FAF-BSA	PPP
effective diffusion coefficient ( <i>D</i> ) cm <sup>2</sup> /sec	$3.05 \times 10^{-11}$	$3.89 \times 10^{-10}$	$7.04 \times 10^{-10}$	$3.89 \times 10^{-10}$
coefficient of determination ( <i>R</i> <sup>2</sup> )	0.972	0.999	0.996	0.998

<sup>a</sup> S1P release data in Figure 6a was least-squares fit to a solution of the unsteady diffusion equation (see text), fitting a single parameter, the effective diffusion coefficient. The release of [<sup>32</sup>P]S1P from PEG-OVS/albumin gels was measured into 0%, 0.4%, and 4% FAF-BSA, as well as into platelet poor plasma (PPP). Additionally, coefficients of determination are reported for linear regressions of S1P release as a function of (time)<sup>1/2</sup>.

The S1P release data were well-described by Fickian release kinetics, given by

$$\frac{m_t}{m_\infty} = 1 - \sum_{n=0}^{\infty} \frac{2}{(n+1/2)^2 \pi^2} \exp[-(n+1/2)^2 \pi^2 D t / b^2] \quad (1)$$

where  $t$  is the time from the start of the release experiment,  $m_t$  is the mass of S1P released up to time  $t$ ,  $m_\infty$  is the mass of S1P ultimately released at infinite time,  $D$  is an effective diffusion coefficient, and  $b$  is the thickness of the gel.<sup>40,41</sup> A single parameter, the effective diffusion coefficient  $D$ , was adjusted to least-squares fit the release data (Figure 6a), considering only the first 100 terms of the infinite series and implicitly assuming that the release solution served as a perfect sink. The effective diffusion coefficients are listed in Table 1.

For short time points (corresponding to less than 60% release), Fickian diffusional release from a thin polymer film can be estimated by<sup>40</sup>

$$\frac{m_t}{m_\infty} = 2 \left[ \frac{D t}{\pi b^2} \right]^{1/2} \quad (2)$$

The release data were described as a function of the square root of time and fit by linear regression (Figure 6b). The coefficients of determination obtained from the linear regressions are listed in Table 1.



The equilibrium affinity constants should be determinable from our experimental system by analysis of S1P release from preloaded gels into PBS or the long-time partitioning of S1P into the gel during postloading. However, these methods would be complicated by the slow release of small amounts of unreacted albumin from the gel (see Figure 2b). Considering the release of S1P from preloaded gels into the PBS washes, the largest fractional release was observed in the first 1 h PBS wash (3.28%), which was more than double that observed with the 0% FAF-BSA wash (i.e., PBS) between 38 and 50 h (1.45%). Using the fractional release into 0% FAF-BSA between 38 and 50 h as an estimate of equilibrium release led to  $K_D = 792$  nM. Further analysis of the effects of  $K_D$  on loading and release of S1P is found in Supporting Information Table 1.

## Discussion

Materials that promote angiogenesis are desirable for treatment of myocardial and peripheral ischemia. A variety of methods of inducing angiogenesis have been explored, including gene therapy,<sup>42</sup> delivery of angiogenic proteins,<sup>43</sup> and angiogenic-potential cell transplants.<sup>44,45</sup> The protein growth factors bFGF and VEGF are the most widely studied angiogenic stimulants. Studies using both of these growth factors have demonstrated induction of in vivo angiogenesis.<sup>14,31,46–48</sup> Initial clinical trials also demonstrated an increase in vascularization.<sup>49–51</sup> However, longer-term clinical studies have shown that newly formed vessels may be unstable, leading to disintegration and little long-term improvement of function.<sup>15,52–55</sup> The long-term failure of therapeutic vascularization is likely due to the rapid depletion of angiogenic factors or the lack of recruitment of mural cells to stabilize the new vessels. With numerous studies reporting this loss of functional vessels after initial vascularization, it is apparent that delivery of factors other than bFGF and VEGF will be necessary to stabilize new vessels.<sup>16</sup> PDGF is one factor that promotes vessel stabilization. Studies utilizing the delivery of multiple growth factors, such as bFGF or VEGF to promote new vessels and PDGF to promote vessel stabilization, have demonstrated an increase in mature vasculature in animal models.<sup>56,57</sup>

Lipid factors such as S1P and lysophosphatidic acid are capable of inducing many events in angiogenesis.<sup>17,19</sup> The primary receptor for S1P ( $S1P_1$ ) has been known to be upregulated in endothelial cells in an angiogenic phenotype since 1990.<sup>58</sup> However, it was only in 1998 that S1P was demonstrated to be the ligand for this G protein-coupled receptor.<sup>59</sup> In particular, S1P is vital to the stabilization of vessels following angiogenesis.<sup>24,60,61</sup> Knockout of  $S1P_1$  is embryonically lethal in mice, because of improper vessel maturation.<sup>25</sup> Since S1P is capable of promoting the earlier steps of angiogenesis, such as endothelial cell migration,<sup>20,27,62</sup> proliferation,<sup>18</sup> and entubulation,<sup>17,19,22</sup> as well as the later steps of vessel maturation, it may be more effective to deliver S1P than combinations of protein growth factors. Furthermore, many studies have found that S1P also serves to protect myocardial cells from cell death induced by ischemia.<sup>63–66</sup>

We demonstrated that the interaction of S1P with albumin immobilized in our materials is necessary to control the rate of S1P release from the PEG hydrogels. However, the specific manner in which the release is controlled is still unknown. Our experiments using 0%, 0.4%, or 4% (w/v) FAF-BSA solutions demonstrated that the concentration of albumin in solution impacts the rate of S1P release. We used S1P release into PPP to simulate release into blood in the body, which has a normal

albumin range of 3.5–4.6 g/dL (3.5–4.6% (w/v)).<sup>67,68</sup> Although 4% albumin is within the normal concentration range of blood, the data for release into PPP indicated that the rate of S1P release into blood may actually be more similar to release into 0.4% FAF-BSA, possibly because of lipids already bound to the plasma albumin. This suggests that the availability of S1P binding sites on albumin (or other lipid carriers) in solution controlled the rate of release, perhaps simply by decreasing the concentration of free S1P at the gel/solution interface. Alternatively, if albumin in solution diffused into the hydrogel during the experiment, the increase in the rate of S1P release in higher concentrations of albumin solution may be explained by the transport of S1P through the gel by the diffusing albumin. In this case, the concentration of dissolved albumin in the gel would be a function of the concentration of albumin in solution and the diffusivity of albumin in the gel. However, modified SDS-PAGE indicated that the diffusion of albumin through the PEG-OVS/albumin gels is greatly restricted (Figure 2b).

If the reaction of a diffusible molecule with an immobilized component is fast relative to the diffusion of the molecule, the diffusion coefficient is effectively lowered by a factor proportional to the fraction of the molecule that is unbound at equilibrium. Thus,  $D_{\text{eff}} = D/(R + 1)$ , where  $R$  is a partition coefficient equal to  $[S1P\text{-}alb_i]/[S1P]$  and  $[S1P\text{-}alb_i]$  is the concentration of S1P bound to the gel.<sup>41</sup> The effective diffusion coefficient can be determined from S1P release data by eq 1, assuming the release solution is a perfect sink. In our system, the effective diffusion coefficient was found to be a function of the concentration of albumin in solution. While loading of S1P onto albumin in PPP, 0.4% and 4% FAF-BSA release solutions is far from equilibrium at every time point (see Supporting Information Table 2), the S1P concentration gradient between the gel and the solution is likely affected by the accumulation of S1P near the interface due to the lack of mixing in the release solution. In Supporting Information Figure 1, we show that the observed differences in effective diffusion coefficients for release into 0.4% and 4% FAF-BSA are explainable by differences in the concentration of free S1P at the boundary in unstirred solutions with  $K_D$  on the order of 100 nM. The perfect sink condition is not achieved with 0.4% or 4% FAF-BSA; however, the error in this assumption is shown to be low for 4% FAF-BSA. Further measurements of the affinity of interactions between albumin for S1P, the rate of diffusion of albumin in the gels, or the amount of unreacted albumin in the gels will be needed to determine the mechanism of S1P release.

The biomaterials we developed may provide a new tool to promote healing and angiogenesis in ischemic tissue. By incorporating the lipid transporter protein albumin into the hydrogels, the release of S1P was controlled by affinity interactions. The high concentration of albumin in the gels (10% w/v, 1.51  $\mu\text{M}$ ) allowed S1P to be efficiently loaded into the gels and slowly released, even into solutions containing high concentrations of lipid transporter proteins. We demonstrated that the S1P released from the hydrogels enhanced migration of endothelial cells growing on the materials and induced angiogenesis in the CAM model. Further development of the materials with higher-affinity lipid-transporting proteins, or enzymes that produce sphingosine 1-phosphate in situ, may lead to materials that are useful in promoting angiogenesis or the endothelialization of materials following implantation.

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**Supporting Information Available.** Further analysis of the S1P release experiment shown in Figure 6. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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