Formation of Microchannels in Poly(ethylene glycol) Hydrogels by Selective Degradation of Patterned Microstructures

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A technique for constructing microchannels within three-dimensional hydrogels is described. The method depends on the use of two photopolymerizable macromers with different degradation kinetics. Poly(ethylene glycol)-diacrylate (PEG-DA) and poly(ethylene glycol)-*co*(L-lactide) diacrylate (PEG-PLLA-DA) were synthesized and characterized by ^{1}HNMR and FTIR. PEG-PLLA (but not PEG-DA) gels degrade rapidly via hydrolysis. Gels were formed via bulk photopolymerization of the macromers by exposure to ultraviolet light in the presence of 2,2-dimethoxy-2-phenylacetophenone (DMPA) as an initiator. Patterns of PEG-PLLA-DA were generated within multilayer PEG hydrogels by noncontact photolithography. When the hydrogels were exposed to high pH conditions, the patterned PEG-PLLA-DA structures degraded rapidly, resulting in channels within the PEG-DA hydrogels. Single and multilayered channels were formed and their structure examined by phase contrast and confocal microscopy.

1. Introduction

The ability to spatially control polymer structure is crucial for many research areas, including microfluidics, tissue engineering, and separation technologies. A number of patterning technologies have been investigated for spatial control of surface chemistry and topography of materials at the micro and submicrometer scale,[1] including photolithography,[2] soft lithography (microcontact printing),[3] microfluidic patterning,[4] and micromolding.[5,6] In general, photolithography is a method to transfer a geometric pattern from a photomask to a light-sensitive chemical on the substrate.[7] Photolithographic techniques allow precise control of topographical features and spatial presentation of desired materials in two dimensions. Most present studies involve patterning on gold and silicon[8] substrates, which are not biocompatible; furthermore, the geometry of many patterning techniques are limited to two-dimensional structures. It is difficult to control spatial features within three-dimensional polymer structures.

Poly (ethylene glycol) (PEG) is a hydrophilic and biocompatible polymer that is readily cleared by the body. PEG is resistant to nonspecific protein adsorption and cell adhesion.[9] Replacing the end-capped hydroxyl groups of PEG with acrylate groups results in a macromer, PEG diacrylate (PEG-DA), that can undergo rapid polymerization in the presence of a suitable photoinitiator.[10,11] Hydrogels formed by photoinitiated cross-linking of PEG-DA have been investigated for a number of biomedical applications[12−20] because of the ease with which biological molecules and cells can be incorporated into the network structure. Hydrogels

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from PEG can be incorporated with cell adhesive peptides such as RGD[21] and YIGSR[22,23] to mimic extracellular matrices. However, PEG hydrogels do not readily degrade under physiologic conditions. Poly (L-lactide) (PLLA) is a hydrophobic and biodegradable polymer that has been studied extensively for drug delivery,[24] bone fixation techniques,[25] and medical device[26] applications. Hydrogels formed from acrylated PEG-PLLA copolymers are degraded under physiologic conditions because of the introduction of biodegradable PLLA units into the polymer backbone.[27]

The generation of microchannels in PEG hydrogels could be used in a number of biomedical applications. Microstructures generated using photolithography on glass have been used for cell sorting.[28] Significant research has been performed where peptides and cells are patterned in hydrogels by using soft photolithography.[29] These techniques may allow for better control over the spatial distribution of cells and proteins in biomaterials. The ability to build three-dimensional interconnected channels in hydrogels may lead to the construction of complex microvascular networks in engineered tissues. However, current techniques are largely limited to the generation of two-dimensional open valleys.

In this research, we developed a technique to construct microchannels within multilayered 3D PEG-DA hydrogels. PEG-PLLA-DA hydrogels were patterned within multilayer PEG-DA hydrogels using noncontact photolithography. Upon exposure to aqueous solution with pH 12, the patterned PEG-PLLA-DA structures were selectively degraded, resulting in microchannels within the PEG-DA hydrogel. With this approach, two and three-dimensional interconnected microchannels could be generated within PEG hydrogels.

2. Experimental Section

2.1. Materials. Poly (ethylene glycol) (average M_n 3400), poly(ethylene glycol) diacrylate (average $M_n \approx 575$), dichloromethane (anhydrous, ≥99.8%), hexane (reagent grade, ≥95%), acryloyl chloride (98%), triethylamine (99.5%), toluene (anhydrous, 99.8%), 3-aminopropyltriethoxysilane (99%), Ti(II) 2-ethylhexanoate (~95%), polished KBr IR crystal windows, triethanolamine (bioUltra, ≥99.5% GC) 2,2-dimethoxy-2 phenylacetophenone (99%), 1-vinyl-2-pyrrolidone (≥99%),and 3,6-dimethyl-1,4-dioxane-2,5-dione were obtained from Sigma (St. Louis, MO). Premium cover glass (size 30 × 22 mm), frosted microscope slides (precleaned), trypan blue stain (4%), and FITC conjugated bovine serum albumin (FITC-BSA) were purchased from Invitrogen (Pittsburgh, PA).

2.2. Synthesis of Hydrogel Precursors. Synthesis of the PEG-PLLA copolymer was based on a procedure previously described by Sawhney et al.[30] and Elbert et al.[31] All glassware and stir bars were cleaned and dried in a vacuum oven at 100 °C for 24 h prior to use. Ten grams of PEG and 2.12 g of *l*-lactide were placed in a dried round-bottom flask and purged with argon for 3 min, and then 40 μL of stannous octoate was added as a catalyst In order to perform the reaction at a uniform temperature the entire flask was submerged in an oil bath. The temperature of the oil bath was increased to 140 °C and the reaction was allowed to proceed for 4 h. The product was filtered twice through 0.45 μm syringe filters. The copolymer was precipitated by dissolving the product in anhydrous dichloromethane followed by precipitation in hexane by slowly dripping the product into hexane while stirring at 3000 rpm.

The PEG-PLLA copolymer was then acrylated as described previously.[30] Ten g of PEG-PLLA were dissolved in 125 mL of anhydrous toluene in a 250 mL round-bottom flask. Water was removed by azeotropic distillation of 50 mL toluene under an argon atmosphere. After the round bottle flask was cooled to room temperature, 25 mL of anhydrous dichloromethane was added to the distilled solution to dissolve the PEG-PLLA. The PEG-PLLA solution was purged with argon for 3 min to remove moisture. Twelve moles of triethylamine (TEA) per mole of PEG-PLLA were added to the round-bottom flask. A syringe pump was used to add acryloyl chloride dropwise. The syringe was filled with 25 mL of dichloromethane and 2.49 mL of acryloyl chloride. The molar ratio of acryloyl chloride to PEG-PLLA used was 10:1. The solution was then stirred overnight in an ice bath. The next day, 25 mL of anhydrous dichloromethane was added to the product, and it was filtered through Whatman 934-AH filter paper and Whatman glass microfiber grade GF/G until the solution was transparent to remove the TEA salt. The products were precipitated in 2 L of hexane to remove the residual acryloyl chloride. The structure and purity of the products were verified by FTIR (Tensor 27 FTIR; Bruker; Billerica, MA) and ^{1}H NMR (Advance 300 Hz; Bruker, Billerica, MA). To perform ^{1}H NMR, we dissolved the products in $CDCl_3$ with 0.05% v/v of tetramethylsilane (TMS) as an internal standard.

2.3. Channel Construction. The hydrogel precursor solution consisted of 23% (w/v) PEG-DA 575 or PEG-PLLA-DA in deionized (DI) water. An initiator solution was made by dissolving 300 mg of 2,2-dimethoxy-2-phenylacetophenone (DMAP) in 1 mL of 1-vinyl-2-pyrrolidone (VP). Three μL of initiator solution was added to the precursor solution, giving a final DMAP concentration of 900 ppm with the molar ratio respect to the number of polymerizable groups being 1:256. Microscope slides and coverslips were used to control the thicknesses of the multilayers formed via bulk polymerization. One hundred μL of PEG-DA (or PEG-PLLA-DA) precursor solution was injected between microscope slides and polymerized under UV light (365nm) for 3 min. The gap between the slides was controlled by coverslips as shown in Figure 1a. Initially, two coverslips were used per side. The coverslips were removed and the first hydrogel layer rinsed with DI water and blotted with kimwipes to remove excess water. Two more coverslips were added to each side, resulting in 4 coverslips between microscope slides (Figure 1b). One hundred microliters of PEG-PLLA-DA precursor was injected on top of the first layer, and a photomask was positioned on the top of the microscope slide (Figure 1c). A small amount of water was added to seal the mask with the slide to eliminate the space between mask and slide. The sample was then exposed to UV light through the photomask for 3 min, generating a strip of PEG-PLLA hydrogel on the surface

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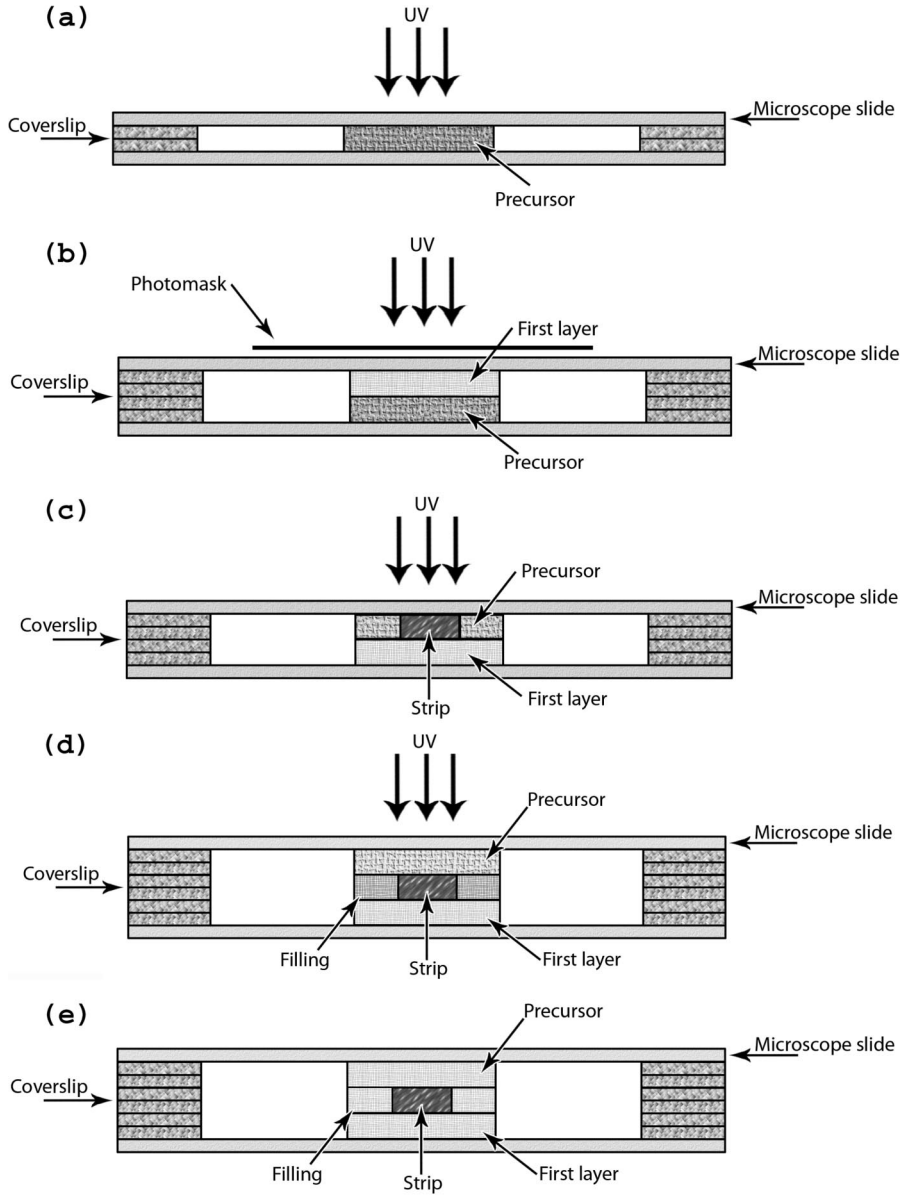

Figure 1. Schematic representation of mulitlayer patterning technique.

of the first hydrogel. In order to fill the spaces between strips in the second layer, the spacers were removed, and the strips rinsed with DI water. The spacers (coverslips) were replaced and 100 μL of PEG-DA precursor injected between the slides. The sample was exposed to UV light for 3 min. The third layer was built having used the same procedure but with an increase in spacers to 6 coverslips (Figure 1d). The same procedure was used to construct multichannels in a single layer, but samples were polymerized for 3 min (Figure 1b). After completion of the multilayer process, the sample was incubated in a high pH environment (pH adjusted to a value of 12 using 1 M NaOH solution) for 3 days to accelerate degradation of the PEG-PLLA-DA copolymer.

A 3D, interconnected channel was patterned by modifying the previous procedure to introduce a second patterned layer. This layer (Figure 1c) contained a perpendicularly patterned PEG-PLLA-DA hydrogel and filled PEG-DA hydrogel resulting in two patterned PEG-PLLA-DA hydrogels interconnected and perpendicular to each other. To demonstrate the 3D structure of the pattern, we incorporated 0.25 mg/mL of FITC-BSA in the PEG-PLLA-DA precursor and imaged the sample by confocal microscopy immediately following synthesis. The first layer and PEG-PLLA-DA precursor with 0.25 mg/mL FITC-BSA was patterned as depicted in Figure 1b. To pattern the perpendicular interconnected PEG-PLLA-DA hydrogel channel, we patterned the second PEG-PLLA-DA hydrogel with FITC-BSA on top of the second layer after the photomask had been rotated 90° (Figure 1b). The procedures for filling each layer and generation of the top layer followed the same procedure described before (Figure 1c). The sample was then imaged by confocal microscopy before it was placed in the controlled pH 12 environment to accelerate PEG-PLLA-DA hydrogel hydrolysis. After the PEG-PLLA-DA hydrogel degraded away, the resultant 3D channels were injected with FITC-BSA (confocal microscopy) and trypan blue (macroscopic imaging) solutions to demonstrate the 3D geometry and connectivity of the resultant channels.

2.4. Confocal Imaging. Confocal microscopy was preformed using a PASCAL Laser scanning microscopy (LSM) system from Carl Zeiss MicroImaging, Inc. (Thornwood, NY). After FITC-BSA was injected into a single channel entrance, the hydrogel was imaged using a 488 nm laser and a 505 nm low pass filter. Images had x and y resolution of 3.5 μm/pixel and z resolution of 20 μm/pixel. The serial images were exported into Axiovision 4.5 (Carl Zeiss) for reconstruction and volume rendering into 3D images.

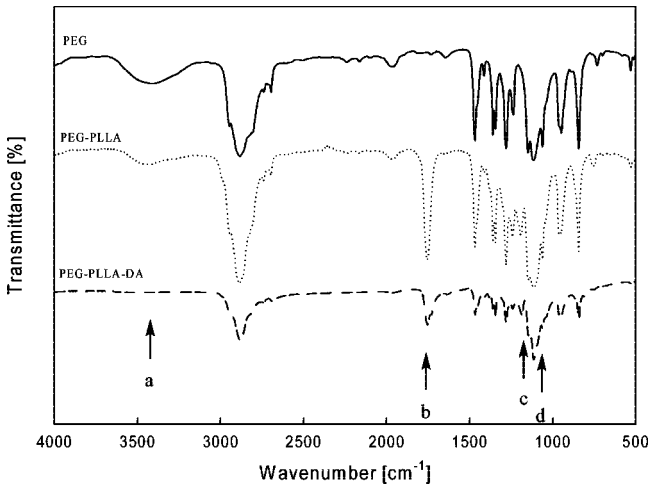

Figure 2. FTIR Spectra of PEG, PEG-PLLA, and PEG-PLLA-DA copolymers. Peaks a−d correspond to vibrations for O−H, C═O, C−C−O, and O−C−C, respectively.

3. Results and Discussion

3.1. Polymer Synthesis. FTIR confirmed the successful synthesis of the PEG-PLLA copolymer. The stretch vibrations of the C═O (peak b), C−C−O (peak c), and O−C−C (peak d) groups in the copolymer are present at 1756, 1185, and 1059 cm^{-1}, respectively (Figure 2). These peaks are characteristic of ester bond absorption and confirm the ring-opening reaction adding lactate to the terminal hydroxyl groups in PEG. After acrylation, the infrared spectrum of PEG-PLLA-DA exhibited almost no absorption band at 3500 cm^{-1} (peak a), which is characteristic of the terminal hydroxyl groups (Figure 2). These results were further confirmed by ^{1}H NMR (vide infra). The results of ^{1}H NMR analysis for PEG-PLLA were as follows. ^{1}H NMR ($DCCl_3$): 3.64 ppm (s, 47.8 H, PEG), 1.58−1.52 ppm (d, 4.75 H, $C\underline{H}_3CH$), 5.20 ppm (q, 1 H $CHCH_3$). The number of repeating units was determined as described by Salvatore et al.[32] PEG-PLLA-DA ^{1}H NMR ($CDCl_3$): 3.64 ppm (s, 169.46 H, PEG), 5.88 ppm (dd, 1.00H, $C\underline{H}_2$═CH−COO−), 6.11 ppm (dd, 1.00H, CH_2═$C\underline{H}$−COO−), 6.33 ppm (dd, 1.12H, $C\underline{H}_2$═CH−COO−). There were unexpected peaks, 1.45 ppm (dd, 0.48H) and 2 ppm(s, 6.79H), that were due to impurities. The contaminants were less than 1%. The method to calculate acrylation efficiency was described by West et al.[33] The acrylation efficiency of PEG-PLLA-DA obtained was 90 ± 3% by using the integral units ratio of summation of 5.88 ppm (dd, 1.00H, $C\underline{H}_2$═CH−COO−), 6.11 ppm (dd, 1.00H, CH_2═$C\underline{H}$−COO−), 6.33 ppm (dd, 1.12H, $C\underline{H}_2$═CH−COO−), to 3.64 ppm (s, 169.46 H, PEG).

The degree of polymerization (DP), calculated from ^{1}H NMR, was determined to be 4.8 lactide groups per mole of PEG macromer by using the integral units ratio of 1.58−1.52 ppm (d, 4.75 H, $C\underline{H}_3CH$) to 3.64 ppm (s, 47.8 H, PEG).

3.2. Microchannels. Hydrogels could be patterned on the surface of a single hydrogel (Figure 1). The thickness of the layer could be adjusted by varying the spacer size and polymerization conditions.[34] According to the manufacturer, the average thickness of a coverslip is between 130 and 170 μm. The first layer (PEG-DA 575) was patterned using 2 spacers, and the strip (PEG-DA 575) patterned using 4 spacers (Figure 3). The thickness of the patterned hydrogel (347 μm) is very close to the thickness of the first layer (392 μm). Both are slightly larger than the thickness of the two hydrogel spacers, possibly due to hydrogel swelling. The patterned structure was wider than the dimensions of the photomask. This most likely results from the use of non-collimated light. The use of collimated light and control of polymerization conditions[34] could improve the agreement between the photomask and patterned structure.

The hydrogel used to fill in the unpatterned region had the same thickness as the patterned structure, resulting in a smooth surface at the interface of the patterned and filled hydrogels (Figure 3b).

To demonstrate multilayer patterning of PEG-PLLA-DA hydrogels, we suspended FITC-BSA (0.25 mg /mL) within the PEG-PLLA-DA precursor solution before patterning. The patterned regions were then imaged by confocal microcopy

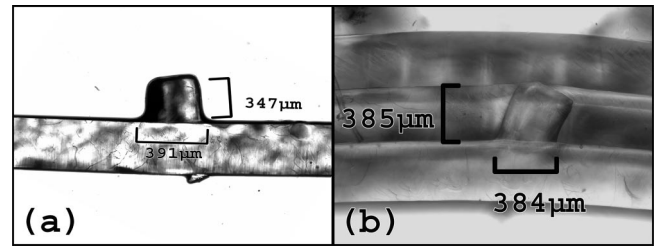

Figure 3. Images of steps in multilayer hydrogel construction. Cross-sectional images showing (a) a PEG-PLLA strip patterned on the surface of a PEG gel, and (b) a multilayer PEG hydrogel with a distinct hydrogel pattern created in the middle layer. Photomask width = 125 μm.

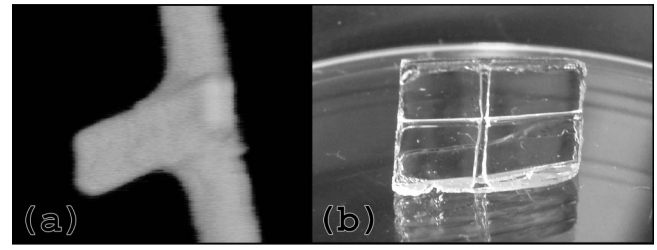

Figure 4. (a) Confocal image of PEG-PLLA hydrogel containing FITC-BSA. The degradable hydrogel was patterned in different planes of the multilayer PEG hydrogel. (b) Two interconnected channels were formed following copolymer degradation.

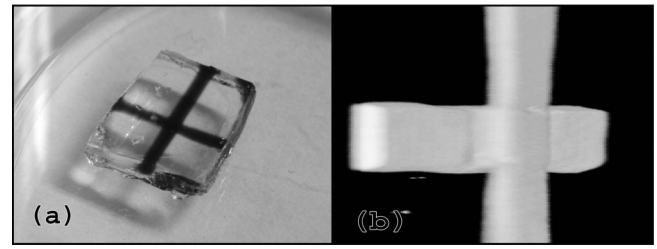

Figure 5. (a) 3D microchannels injected with trypan blue. (b) Three-dimensional reconstruction of confocal imaged of FITC-BSA injected into the microchannels.

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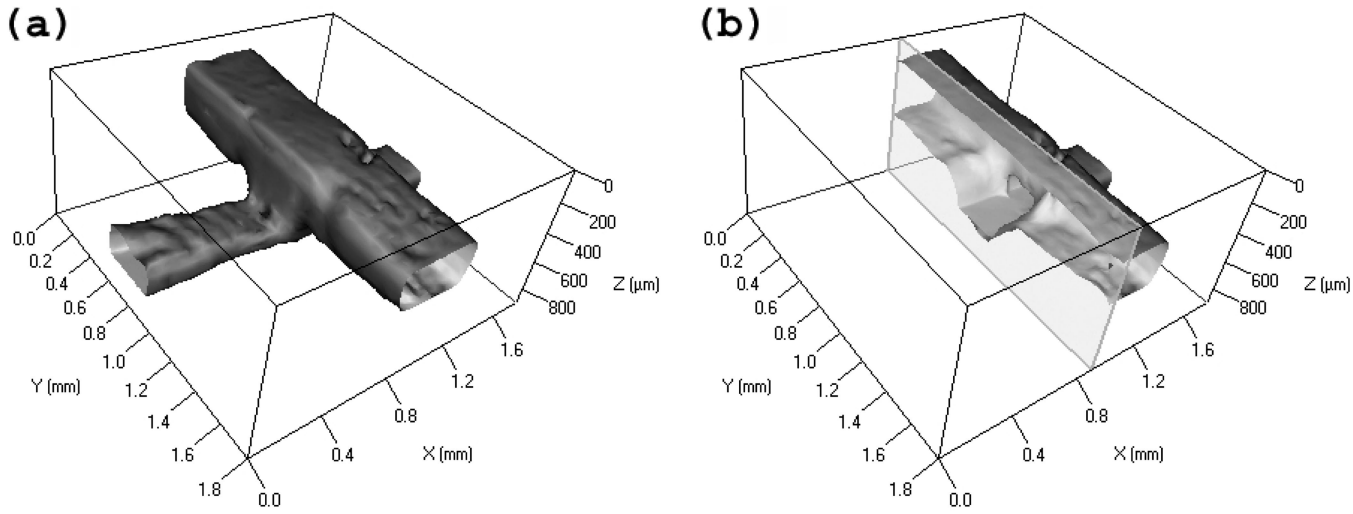

Figure 6. (a) Three-dimensional projection from confocal images of microchannels filled with FITC-BSA. (b) Cross section of the 3D projection along the $x-z$ plane.

(Figure 4a), revealing two layers of PEG-PLLA-DA strips that intersected at one point. After incubating the sample for 3 days to degrade the PEG-PLLA-DA patterns, interconnected 3D channels were left within the hydrogel (Figure 4b). As a demonstration of the connectivity of the patterned channels, trypan blue was injected into the end of a single channel, where it then filled both channels completely by capillary action (Figure 5a and the video in the Supporting Information). As additional evidence of the 3D structure of the multichannels, FITC-BSA was injected into the channels and imaged with confocal microscopy (Figure 5b). Three-dimensional projections of microchannels filled with FITC-BSA were produced showing the entire structure (Figure 6a) and a cutting plane along the $y-z$ plane showing the interconnected channels (Figure 6b). The images reveal that the technique was able to produce multilayer interconnected channels.

Microchannel generation by selected degradation of patterned PEG hydrogels has several advantages over existing methods for generating channels. First, the three-dimensional architecture with interconnected microchannels was obtained by this technique, and other techniques create a single layer of channels on flat surfaces.[35−37] Second, microchannels were created in PEG substrates. PEG is biocompatible and allows for easy incorporation of various peptides and proteins for engineering scaffolds with distinct biologic activity.[38,39] The ability to create channels within PEG hydrogels could be used to pattern unique structures, such as branching networks similar to those found in microvasculature, that may be required for clinical success of engineered tissues.[40] Other methods have been used to generate microchannels in nonbiocompatible substrates such as silicon.[28] These materials cannot be directly implanted into the body. In addition, those approaches do not allow for the creation of multilayer structures as shown here.

The lateral resolution of the patterns formed was determined by the photomask and polymerization conditions, whereas the thickness of the layers microstructure was determined by spacers between the two glass slides. Coverslips were used as spacers and they allowed for good control between thickness of the patterned and filled regions of hydrogels in a single layer. However, the spacers limit the thickness of the layers that can be formed. In previous studies, interfacial polymerization has been shown to provide good control over the thickness of hydrogels formed on the basis of the polymerization conditions including exposure times and the power density of laser.[34] The microfabrication technique described here could be further refined using interfacial photopolymerization to allow more versatility in the thickness of the patterns that can be formed.

The patterned structures were degraded by incubation at high pH. Three days were required for complete degradation of the patterned copolymer. The goal is to modify these microchannels with protein or peptide sequences to study cell migration and proliferation.[41] The degradation of the copolymer is not expected to alter cell function and migration. Hydrogels formed from PEG-PLLA copolymers have been used in a number of biomedical applications with little evidence of altered cell behavior.[42] The high pH used during the degradation step may influence the biologic activity of proteins or peptides. Degradation of PEG-PLLA can also be performed at physiologic pH (7.4), which would not be expected to be detrimental to protein activity. However, at pH 7.4, the time required for degradation will be around 27 days (data not shown). The degree of polymerization of the copolymer could also be altered to allow for more rapid degradation of the patterned structures.

PEG hydrogels photopolymerized in the presence of DMPA has been used for encapsulation of viable cells,

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including smooth muscle cells[38] and endothelial cells,[39] However, in cell culture studies, DMPA has toxicity when incubated directly with cells. If the channels are to be seeded with cells postsynthesis, then residual DMPA can be extracted prior to seeding. In addition, other low-toxicity photoinitiators such as 1-phenyl-2-hydroxy-2-methyl-1-propanone[28] and 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanon[43] could be used with the same protocol.

Conclusions

A technique was developed in which microchannels can be created within PEG hydrogels by selective degradation of patterned PEG-PLLA-DA copolymer structures. The technique can be used to generate 3D interconnected channels. Currently, we are investigating whether microchannels incorporated with cell adhesion sequences can be seeded with endothelial cells to build vascular networks.

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Supporting Information Available: Two film clips (WMV). This material is available free of charge via the Internet at http://pubs.acs.org.

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