Langmuir
The ACS Journal of Surfaces and Colloids

JUNE 22, 2004
VOLUME 20, NUMBER 13

Letters

Fabrication of Gradient Hydrogels Using a Microfluidics/Photopolymerization Process

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Received March 17, 2004. In Final Form: April 30, 2004

A method of fabricating photo-cross-linked hydrogels with gradients of immobilized molecules and cross-linking densities is introduced. Two macromer/initiator solutions are injected into a unique poly(dimethylsiloxane) channel system that produces a prepolymer gradient that is subsequently polymerized into a water-swollen hydrogel with ultraviolet light exposure. The gradient is controlled by the injection flow rate (optimized to 0.3 μL/min per inlet to produce a linear gradient). The technique is investigated both through fabrication of adhesive ligand gradients that modulate spatial distribution of attached endothelial cells and gradients of cross-linking densities that led to unique hydrogel architectures and spatially dependent swelling.

Introduction

Cell migration is controlled by gradients of soluble (chemotaxis) and immobilized (haptotaxis) molecules and substrate mechanics (durotaxis).[1,2] For example, neuron growth cones are guided to various termination sites by gradients of guidance cues.[3] There have been numerous studies to investigate the influence of these gradients of molecules and cues on specific cellular behavior (e.g., migration or axon orientation), but these approaches have relied on complex and experimentally intensive techniques for gradient production.[4−6] Thus, it is of interest to develop techniques to fabricate model substrates that can be used to investigate these complex cell behaviors in vitro to provide information that can potentially be used for the development of therapies, such as scaffolding for tissue engineering.

Hydrogels are water-swollen polymer networks that are being investigated for drug delivery and tissue engineering[7,8] as a result of their biocompatibility and controllable properties (e.g., mechanics and degradation). For instance, poly(ethylene glycol) (PEG) hydrogels are inherently cell-repellant (as a result of poor protein adsorption), and, thus, cell interactions with PEG hydrogels are controlled by the incorporation of molecules without nonspecific protein adsorption interfering.[9] Additionally, the physical properties of PEG hydrogels are controlled by monomer properties such as the molecular weight of the PEG or the macromer concentration[10] to provide simple techniques

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10.1021/la049298n CCC: $27.50
Published on Web 05/28/2004

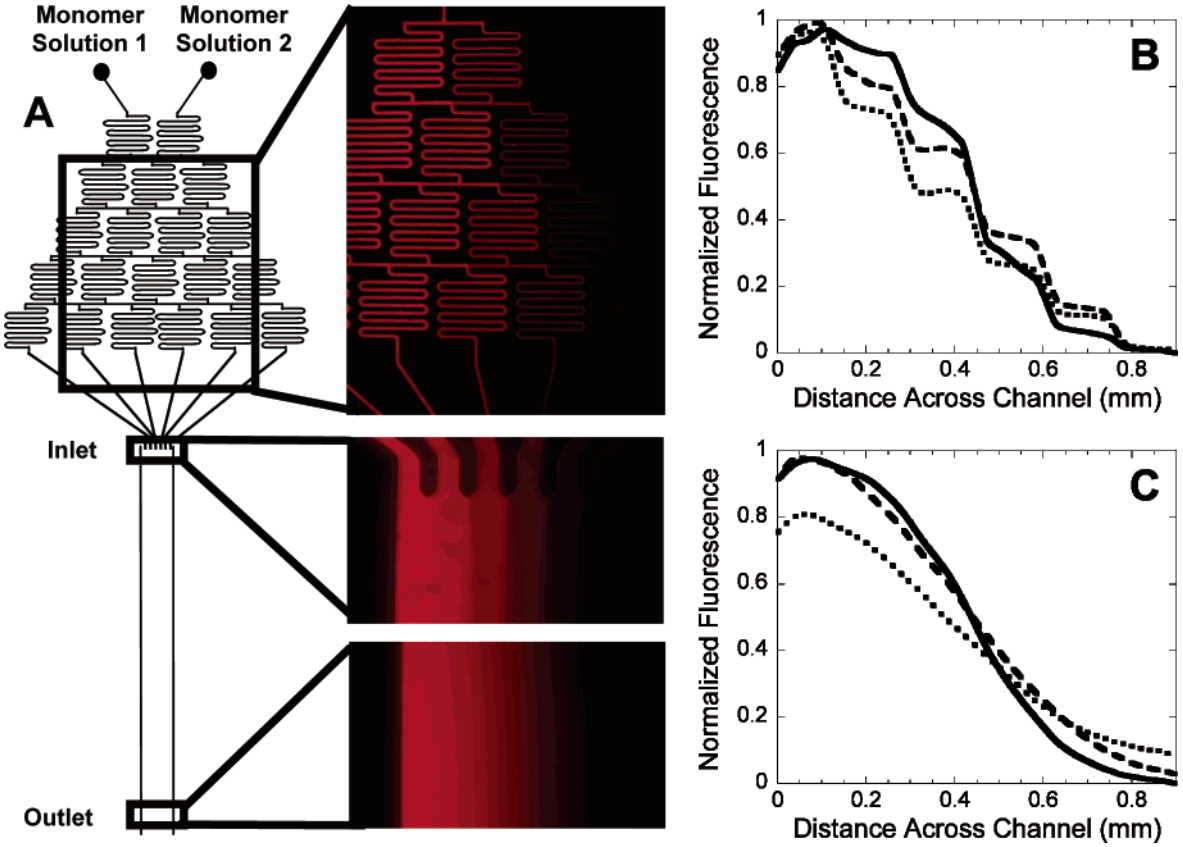

Figure 1. Schematic of the channel used in the microfluidics/photopolymerization process (A) along with fluorescent images of the gradient maker and channel gradients at the inlet and outlet (~20 mm downstream of the inlet), where rhodamine is incorporated into monomer solution 1 and the monomer solutions are flowed at a rate of 0.3 μL/min. Gradient quantification at the inlet (B) and outlet (C) for monomer solution flow rates of 1.0 μL/min (solid line), 0.3 μL/min (dashed line), and 0.05 μL/min (dotted line).

to alter the network cross-linking density. In this work, we have utilized microfluidic-based systems incorporating a unique gradient maker channel that can be used to fabricate PEG-based hydrogels with gradients of tethered molecules and hydrogel cross-linking densities.

Experimental Methods

Poly(ethylene glycol)-4000 diacrylate (PEG4000DA) and acryloyl-poly(ethylene glycol)-RGDS (Acr-PEG-RGDS) were synthesized as described previously,[9,10] and the acrylation efficiency (>95%) was confirmed with ^{1}H NMR. Briefly, Acr-PEG-RGDS was synthesized by the reaction of acryloyl-PEG-*N*-hydroxysuccinimide (3400 Da, Nektar Therapeutics) with GRGDS (Bachem) in 50 mM sodium bicarbonate buffer (pH 8.2) for 2 h at room temperature. All chemicals were purchased from Aldrich and used as received unless noted otherwise. Macromers were dissolved in phosphate buffered saline (PBS) at desired concentrations, and 0.5 wt % 2-hydroxy-2-methyl propiophenone (a water-soluble photoinitiator) was added. Glass slides were modified with methacrylate functional groups for immobilization of polymerized hydrogels by immersing slides in a solution of 30% H_2O_2 and H_2SO_4 (3:1 ratio) for 5 min, washing in DiH_2O, immersing in 10 mM 3-(trichlorosilyl)propyl methacrylate (TPM), and washing with heptane/carbon tetrachloride and DiH_2O.[11] Poly(dimethylsiloxane) (PDMS) molds were fabricated by curing prepolymer (Sylgard 184, Essex Chemical) on silicon masters patterned with SU-8 photoresist.

Microfluidic devices were constructed by placing PDMS molds on the TPM slides and plasma cleaning (PDC-001, Harrick Scientific Co.) with the lower portion of the slides covered to aid in the future detachment of the mold from the slide after hydrogel formation. The general procedure for gradient hydrogel formation involves the injection of two monomer solutions into a unique gradient maker that consists of a network of microchannels that repeatedly split and mix the injected solutions.[12] This channel system was previously used to investigate cell interactions with solution[12] and substrate-bound[13] gradients. After passing through the gradient maker, the monomer solutions enter a larger viewing channel, where a stable gradient is formed (visualized with a fluorescent microscope where one monomer solution contained 1 wt % rhodamine), and the solutions are photopolymerized into a hydrogel with ultraviolet light exposure (~200 mW/cm^2 for 60 s, EXFO Spot Curing System) through the PDMS mold. Although the rhodamine molecule is smaller than the injected PEG macromers, rhodamine fluorescence should give a good estimate of gradient production in the microchannel. The mold is then removed to leave a hydrogel structure (~80 μm in height) immobilized on a glass slide. A similar procedure was used by Pishko and co-workers[14,15] to develop nongradient hydrogel microstructures.

Human umbilical vein endothelial cells (HUVECs, Clonetics) were maintained in an endothelial cell basal medium supplemented with the supplied Bulletkit (Clonetics). Upon trypsinization, cells were seeded on the hydrogel microstructures (washed several times in PBS and sterilized with ultraviolet light exposure in a laminar flow hood) at a density of ~300 cells/mm^2. After 3 h, the hydrogels were rinsed with PBS, fixed in 2.5% glutaraldehyde (Polysciences) for 15 min, washed in PBS, and visualized (Zeiss Axiovert 200 inverted microscope). Cells were quantified

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by counting attached endothelial cells in a minimum of five images from three individual hydrogels.

Monomer solutions of various macromer concentrations (10−50 wt %) and macromer molecular weights (PEG1000 to PEG4000) were used to fabricate hydrogel microstructures with gradients in hydrogel mechanics. Samples were air-dried, cut with a scalpel blade to obtain a cross section, sputter-coated with gold, and imaged using a JEOL 6320FV scanning electron microscope. Additionally, the change in cross-linking density across the gradients was visualized and quantified by the diffusion of rhodamine (1 wt %) entrapped in the hydrogel networks during polymerization and subsequent release into PBS.

Results and Discussion

Gradient hydrogels were fabricated through a microfluidics/photopolymerization process which is detailed in Figure 1A. At the inlet to the viewing channel, the microchannel solutions merge and a step distribution of fluorescence is seen as a result of the incorporation of rhodamine in the inlet solutions. This distribution was quantified using NIH imaging software and is plotted in Figure 1B for initial injection rates of 0.05, 0.3, and 1.0 μL/min. Although there are slight differences in the profiles, the step behavior is seen with all flow rates at the inlet. However, under proper flow rates the step gradient quickly formed a smooth gradient down the channel. At the outlet (~20 mm downstream from the inlet), the solutions have mixed and a more linear gradient is seen (fluorescent image in Figure 1A and quantification in Figure 1C). For these systems, a flow rate of 0.3 μL/min per inlet produced the best (most linear) gradient at the outlet. If the flow rate was too fast (i.e., 1.0 μL/min), there was not enough mixing and the gradient plateaus near the edges of the channel, whereas if the flow rate was too slow (i.e., 0.05 μL/min), there was too much mixing and the extremes of the gradient are minimized. These results indicate that the monomer solution flow rate is important to the solution mixing in the channels and that through the appropriate flow rate selection, a near linear gradient at the outlet is observed. Although our goal in this work was the formation of linear gradient profiles, complex profiles (e.g., step gradients, multiple peaks) could be obtained by varying the number of inlets or solution flow rates.[12]

As an initial examination of this gradient process, adhesive ligands (e.g., RGDS) were tethered throughout the hydrogel networks via a PEG spacer. For gradient hydrogels, the Acr-PEG-RGDS was mixed in monomer solution 1, whereas monomer solution 2 only contained the PEG4000DA. HUVECs were seeded on the surface of hydrogel microstructures without RGDS (Figure 2A), with RGDS (Figure 2B), or with a gradient of tethered RGDS (Figure 2C) and visualized. A concentration of 5.0 mM Acr-PEG-RGDS was chosen because of preliminary results of HUVEC attachment on PEG hydrogels with a range of RGDS (results not shown). On unmodified hydrogels, very few HUVECs attached and there was little spreading of the few attached cells because of the lack of protein adsorption on the hydrophilic surfaces. When RGDS was tethered to the hydrogels, HUVEC attachment and spreading was seen across the entire hydrogel microstructure. As expected, HUVEC attachment varied spatially across the hydrogels when the RGDS was tethered in a gradient. The HUVECs also appear to spread better toward the high RGDS side of the hydrogel and were more rounded toward the unmodified RGDS side. As further evidence of cell adhesion on the surfaces of the hydrogel microstructures, the cells attached on the surrounding glass slides are out of focus and, thus, on a different focal plane. Cell migration into the PEG hydrogels was not seen throughout the experiments, but future studies could also use this process to characterize cell migration through enzymatically degradable hydrogel networks with gradients of adhesive tethers or hydrogel cross-linking.

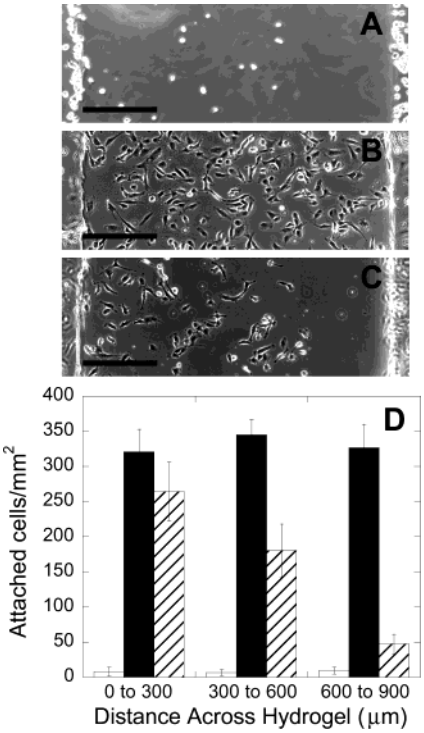

Figure 2. Light micrographs of endothelial cells attached (3 h after seeding) to the surface of hydrogels fabricated without RGDS (A), with 5.0 mM Acr-PEG-RGDS (B), and with a gradient from 5.0 mM Acr-PEG-RGDS (left) to no RGDS (right; C); bar = 200 μm. Quantification of spatial endothelial cell attachment (number of cells/mm^2 hydrogel surface) on 300 μm sections of hydrogels fabricated without RGDS (white), with 5.0 mM Acr-PEG-RGDS (black), and with a gradient from 5.0 mM Acr-PEG-RGDS to no RGDS (striped; D).

When quantified (results in Figure 2D), the same trend was seen with higher cell attachment on the high RGDS side and less attachment on the lower to no RGDS side. For instance, the attachment on the left third of the gel was ~224 cells/mm^2, ~181 cells/mm^2 for the middle third, and ~47 cells/mm^2 for the right third. As expected, there was little difference in spatial cell adhesion on nongradient hydrogels (either unmodified or completely modified with RGDS).

Another potential benefit of this fabrication process is that hydrogels with gradients in cross-linking densities are easily produced by incorporating solutions of various molecular weights and macromer concentrations, which can influence the overall cross-linking density of the resulting hydrogel. This was investigated by injecting a solution of 10 wt % PEG4000DA (low macromer concentration, high macromer molecular weight) into one of the inlets and a solution of 50 wt % PEG1000DA (high macromer concentration, low macromer molecular weight) into the other inlet. After polymerization, these channels were dried and cut to visualize the channel cross sections using scanning electron microscopy (SEM). The SEM images in Figure 3A,B show that the control sample using only the 10 wt % solution produces a very thin network after drying (~10 μm); whereas, the 50 wt % solutions produces a network ~50 μm in thickness. The gradient hydrogel, shown in Figure 3C, produces a sloped network

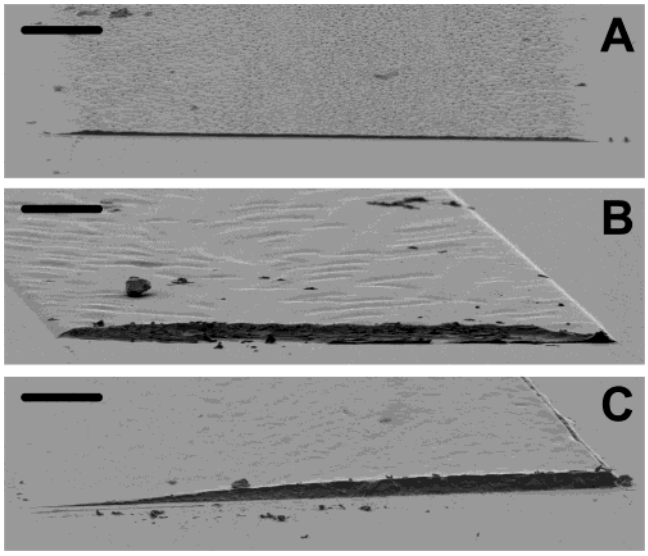

Figure 3. SEM micrographs of cross sections of dried hydrogels fabricated from 10 wt % PEG4000DA (A), 50 wt % PEG1000DA (B), and a gradient of 10 wt % PEG4000DA (left) to 50 wt % PEG1000DA (right; C); bar = 100 μm.

with the thickness varying from left (low cross-linking density) to right (high cross-linking density). It should be noted that a large difference in the viscosities of the injected macromer solutions may influence the final hydrogel gradient and, thus, is a limitation to this technique depending on the magnitude of differences in the macromer molecular weights and concentrations.

As an additional measure of the gradients in the cross-linking density, a fluorescent dye was photoencapsulated in the hydrogel. The hydrogel was then imaged immediately after polymerization (Figure 4A) and after 20 min of swelling in PBS (Figure 4B). A decrease in the fluorescence on the side of the hydrogel that is more loosely cross-linked (greater mesh size) indicates swelling and release of the fluorescent molecule from the network. On the more densely cross-linked side (smaller mesh size) a larger fraction of the fluorescent molecule remains entrapped. The fluorescent intensity was quantified for each image using NIH image software and showed an ~77% decrease in intensity after swelling for the more loosely cross-linked side, whereas only an ~22% decrease was seen on the more highly cross-linked side.

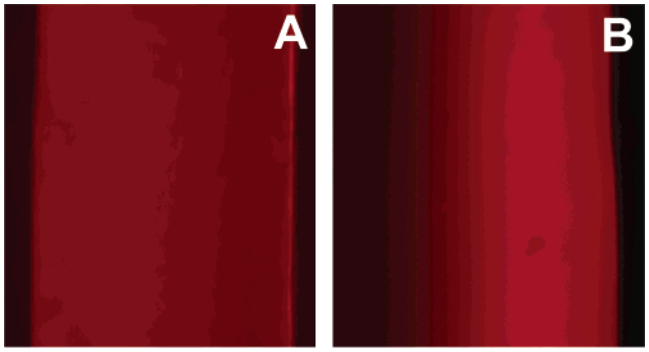

Figure 4. Fluorescent micrographs of rhodamine encapsulated in a gradient hydrogel of 10 wt % PEG4000DA (left) to 50 wt % PEG1000DA (right) immediately after polymerization (A) and after 20 min of release in PBS (B), illustrating gradient-dependent diffusion with greater amounts of rhodamine released from the less cross-linked side of the hydrogel structure and little release from the more cross-linked side (channel width = 900 μm).

In summary, we have introduced a simple technique for the fabrication of gradient hydrogels using a microfluidic system in combination with a photopolymerization reaction. Although not a focus of this study, future work could include the photoencapsulation of cells and molecules in these gradient hydrogel microstructures to produce unique tissue engineering scaffolds. For example, investigators have encapsulated cells such as HUVECs,[16] fibroblasts,[15] and *Escherichia coli*[17] previously using microfluidic channels, although not in gradients.

Acknowledgment. The authors would like to acknowledge funding through the MIT Institute for Soldier Nanotechnology and a fellowship from the Paralyzed Veterans of America Spinal Cord Research Foundation (2268-02) to J.A.B. and David LaVan and Jeffrey Karp for helpful discussions.

LA049298N

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