Rapid Generation of Biologically Relevant Hydrogels Containing Long-Range Chemical Gradients

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Many biological processes are regulated by gradients of bioactive chemicals. Thus, the generation of materials with embedded chemical gradients may be beneficial for understanding biological phenomena and generating tissue-mimetic constructs. Here a simple and versatile method to rapidly generate materials containing centimeter-long gradients of chemical properties in a microfluidic channel is described. The formation of a chemical gradient is initiated by a passive-pump-induced forward flow and further developed during an evaporation-induced backward flow. The gradient is spatially controlled by the backward flow time and the hydrogel material containing the gradient is synthesized via photopolymerization. Gradients of a cell-adhesion ligand, Arg-Gly-Asp-Ser (RGDS), are incorporated in poly(ethylene glycol)-diacrylate (PEG-DA) hydrogels to test the response of endothelial cells. The cells attach and spread along the hydrogel material in a manner consistent with the RGDS-gradient profile. A hydrogel containing a PEG-DA concentration gradient and constant RGDS concentration is also shown. The morphology of cells cultured on such hydrogel changes from round in the lower PEG-DA concentration regions to well-spread in the higher PEG-DA concentration regions. This approach is expected to be a valuable tool to investigate the cell–material interactions in a simple and high-throughput manner and to design graded biomimetic materials for tissue engineering applications.

1. Introduction

Interactions between cells and materials have been shown to profoundly regulate the characteristics, such as morphology, adhesion, locomotion and gene expression. Thus, the ability to recreate and study such interactions may facilitate the development of bioactive and biomimetic materials for various biological and biomedical applications. Numerous studies have shown that both chemical and physical properties of the matrix materials are important to determine the cellular behavior in the surrounding natural or artificial microenvironments. It should be noted that most previous investigations of the cell–material interactions have used individual material samples with uniform chemical or physical properties, which limits the number of samples for testing and their application in studies that require continuous variations in the material properties (chemical or physical), such as haptotaxis or durotaxis. Most of the biomaterials designed for tissue-engineering applications also lack the spatially and structurally defined anisotropic properties that exist in native tissues. Biomaterials with a continuous variance in properties are, therefore, of great interest to create biomimetic cellular niches for biological investigations and tissue-engineering applications.

Numerous approaches have been adopted to engineer material gradients with chemical variance, including manipulating the diffusion pattern or duration of exposure of the photocrosslinkable materials to UV or laser irradiation, electrochemistry, plasma polymerization, fluidic gradient mixers and microfluidic gradient generators. However, most of the approaches mentioned above require long and sophisticated fabrication procedures involving expertise and expensive equipment. In addition, it is normally difficult to generate long-range material gradients, which will increase the number of conditions and accuracy for the quantitative investigation of cell–material interactions, and graded materials for tissue engineering applications. For example, to generate long-range hydrogel gradients by pure diffusion, it may take several days to establish a gradient of the desired molecules from the source to the sink before polymerization. In the case of microfluidic gradient generators, the length of the gradient is determined by the combined length of all the input streams. Thus, in order to generate a centimeter-long gradient, a complicated microfluidic network containing a large number of conditions and accuracy for the quantitative investigation of cell–material interactions, and graded materials for tissue engineering applications.

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of input streams will be required. Such technical difficulties may be the reason that previous microfluidic-based techniques have been limited to generating gradients that were only a few hundred micrometers in size.

We have developed a rapid and simple microfluidic-based approach to generate gradient of chemical properties within biologically relevant hydrogels. By integrating portable microfluidic manipulation and photopolymerization, a hydrogel gradient can be rapidly achieved with minimal requirements in cost or expertise. The size of such gradients is on the centimeter length scale, which has been difficult to achieve by most of the existing methods. Our approach for making materials with embedded chemical gradient is based on using a portable microfluidic device for generating a molecular concentration gradient, which is initiated by a passive-pump-induced forward flow and further developed during an evaporation-induced backward flow. Here we engineered spatially controlled centimeter-long hydrogels by initially generating a concentration gradient of photo-crosslinkable hydrogel precursors, which contain a mixture of PEG-DA, photoinitiator, and/or PEG derivative of the cell-adhesive peptide RGDS (acyrlyl (Acr)-PEG-RGDS) in the microfluidic device. The concentration gradient (either Acr-PEG-RGDS gradient or PEG-DA gradient) was photopolymerized later to form the material gradient with variance in either RGDS concentration or PEG-DA concentration. This microfluidic-based material gradient approach enables the rapid and easy generation of biomaterials with a continuous variance in concentration, cell adhesiveness, and porosity, which is envisioned to be a powerful tool for a wide range of biological and tissue-engineering applications.

2. Results

The gradient-generation and stabilization processes are illustrated in Figure 1. The gradient materials were generated inside a fluidic channel that was formed by reversibly sealing a polydimethylsiloxane (PDMS) mold, containing the impression of the channel, onto a glass slide (Fig. 1A). The channel was first pre-filled with a low-concentration PEG-DA precursor from the outlet and a high-concentration PEG-DA precursor containing the chemicals of interest were introduced from the inlet (Fig. 1B). The solution of interest flowed spontaneously into the channel by a passive-pumping-induced forward flow. The gradient was generated by the combined effect of evaporation-induced backward flow and molecular diffusion. The solution of interest flowed spontaneously into the channel by a passive-pumping-induced forward flow. The gradient was generated by the combined effect of evaporation-induced backward flow and molecular diffusion. The backward flow was stopped by placing the fluidic system into a humidified chamber, and subsequently the hydrogel precursors were photopolymerized to form the gradient hydrogel (Fig. 1D). As shown in Figure 1F, the resultant hydrogel is comprised of three different regions: 1) a positive control region with bioactive chemicals of interest or the highest PEG-DA concentration, 2) a centimeter-long gradient region, and 3) a negative control region with no bioactive chemicals of interest or the lowest PEG-DA concentration.

Several factors were essential to create the centimeter-long gradient hydrogel. First, the PDMS mold should be hydrophobic to maintain the spherical shape of the droplets, which is critical to enable the passive-pumping process. Therefore, the PDMS mold should not be plasma treated. Second, the length of the gradient could be tuned by controlling the backward flow time. As shown in Figure 2A, when the solution was fully introduced into the channel, the flow profile was parabolic and the length of the gradient was about 4 mm. The parabolic profile gradually flattened in the first 5 min because of the evaporation-induced backward flow. As the backward flow continued, a centimeter-long
control region was $93 \pm 7$ cells mm$^{-2}$, and the cell spreading area was $842 \pm 136$ $\mu$m$^2$, while at the negative control region, there were few cells and the cell spreading area was $271 \pm 41$ $\mu$m$^2$. For the gradient region, the larger error bars indicate that the HUVECs are distributed in a gradient manner, which is further validated by the quantification of the cell number in the gradient region (Fig. 5G). Moreover, the concentration of RGDS tethered to the hydrogel also affected the cell-spreading area as shown in Figure 5H.

To investigate the effect of the PEG-DA concentration gradient on cell behavior, we generated gradient hydrogels ranging from 30 wt % PEG-DA to 5 wt % PEG-DA with a uniform concentration (5.0 mM) of Acr-PEG-RGDS. As shown in Figure 6A, the cell morphology in the different regions of the hydrogel was significantly different. In the negative control region (5 wt % PEG-DA), the cells were round in shape and did not spread. In contrast, in the positive control region (30 wt % PEG-DA), the cells were well-spread and appeared to be well-adhered to the underlying substrate. Most notably, the morphology of the cells located in the gradient regions gradually changed from round to well-spread. We also quantified the cell-spreading area in the three regions of the hydrogel. The results (Fig. 6B) show that the average cell-spreading area in the gradient region was $705 \pm 273$ $\mu$m$^2$ and those for the positive control and negative control regions were $662 \pm 71$ $\mu$m$^2$ and $316 \pm 19$ $\mu$m$^2$, respectively. In the gradient region the cell-spreading area gradually increases in a gradient manner (Fig. 6C). However, it should be noted that the cell number attached to the hydrogel increased with a decrease in PEG-DA concentration, which was also observed for cells that were cultured on hydrogels with constant PEG-DA concentrations (5 wt %, 15 wt %, and 30 wt%) (see Supporting Information).

3. Discussion

The goal of the present study was to develop a simple, rapid, and versatile approach for the generation of stable long-range gradient hydrogels using a microfluidic method. Several features of the current approach for gradient generation distinguish it from existing methods: 1) the resultant hydrogel gradient has three different regions: a positive control, a negative control, and a gradient region; which provides a high-throughput platform for biological studies with little experimental error; 2) the size of the hydrogel gradient is on the centimeter length scale; 3) the generation of the gradient is rapid (within 30 min), highly dynamic (through the backward flow stage), and spatially/temporally controllable (by controlling the evaporation-induced backflow); 4) the gradient can be formed by using only low amounts of the molecules of interest (about 2 to 6 $\mu$L for the current microfluidic channel design); and 5) the approach is simple and highly reproducible in a portable microfluidic device, requiring only a pipette for implementation.

We have shown that to maintain the integrity of the hydrogel gradient, the concentration of pre-filling PEG-DA solution should be at least 5 wt % for photopolymerization. On the other hand, to generate the gradient profile in the final hydrogel as predicted by using rhodamine, the concentration of PEG-DA solution should be less than 40 wt %. Higher concentrations of PEG-DA solution were too viscous to fill the microfluidic channel by our surface-tension driven process. However, it is possible to use higher concentrations by simple modifications to the experimental procedures, such as, by using a syringe pump. In this study, we have successfully created a gradient hydrogel with 40 wt % and 5 wt % PEG-DA concentrations. When the
hydrogel was freeze dried, a porous scaffold was generated with gradient porosity. This may be of particular importance for engineering tissues with gradient porous structures and material distributions, such as cartilage.\(^{[20]}\) It can be envisioned that a functional scaffold with different biomaterial distribution and gradient porous structures could be fabricated by combining the presented gradient-generation method with a freeze-drying method. Moreover, it is possible to incorporate bioactive molecules or proteins (e.g., growth factors) into the porous scaffolds in an advantageous manner.

Cellular behaviors, such as adhesion and spreading, are largely regulated by the presence of adhesive molecules. To address these issues, we first generated a RGDS gradient in the cell-repellent PEG-DA hydrogels. The HUVECs cultured on the chemical gradient hydrogel show dramatically different responses dependent on whether they are located on the positive control, the gradient, or the negative control region in the same hydrogel. Especially in the gradient region, the cell number gradually increased with the addition of RGDS peptide.

For the hydrogels with the gradient PEG-DA concentrations, we found that the morphology of the cells located on the gradient regions gradually changed from round to well-spread, which corresponds well with what has been reported previously for vascular smooth muscle cells cultured on hydrogels with a concentration gradient of polycrylamide.\(^{[20]}\) However, the cell number attached on the hydrogels showed an opposite trend in comparison to the cell-spread area. The negative effect of PEG-DA concentration on cell attachment might be attributed to the blocking effect of PEG-DA on the RGDS ligand exposed to cells cultured on the hydrogel. Since PEG is well known for its blocking effect on bioactive surfaces, higher PEG-DA concentrations may lead to a relatively lower RGDS ligand-density exposure to cells on the hydrogel surface.

4. Conclusions

We have presented a simple and versatile approach to rapidly generate biologically relevant hydrogels with a chemical gradient by combining passive-pump induced forward flow, evaporation-induced backward flow, and photopolymerization techniques in a microfluidic channel. The parameters affecting the generation of a gradient hydrogel were investigated, such as the backward flow time and the lowest concentration of pre-filling PEG-DA solution. The resultant hydrogel contains a positive control region, a gradient region, and a negative control region, which makes the experimental results more accurate and comparable. Porous tissue-engineering scaffolds with gradient porosity variation could be generated by freeze drying the hydrogel with the PEG-DA concentration gradient. The response of endothelial cells (adhesion and spreading) to the resultant chemical gradient hydrogels further validated that the presented gradient-hydrogel-generation method is a promising platform to study biological processes such as cell migration and cell–material interactions.

5. Experimental

**Materials:** PEG-DA (M, 4000) was purchased from Monomer-Polymer & Djac Labs and Acr-PEG-RGDS was synthesized as described previously \(^{[31,32]}\). Briefly, Gly-Arg-Gly-Asp-Ser (GRGDS) (1 mg mL\(^{-1}\), Bachem) was reacted with an equimolar amount of acrylate-PEG-N-hydroxysuccinimide (3500 Da, Jenkem Technology) in a sodium bicarbonate buffer (50 mM, pH 8.2) for 2 h at room temperature. The product was dialyzed, freeze dried, and stored at −20 °C until use. The photoinitiator was 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (I2959, Ciba Geigy, at a concentration of 0.5 wt %). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specifically mentioned.

![Figure 5](image-url)
Figure 6. Hydrogels with PEG-DA concentration gradient and constant RGDS concentration to guide HUVECs spreading. A) Representative phase-contrast images to show the cell morphologies at different regions of a 5 cm-long hydrogel (scale bar: 200 μm). B) Quantification of the cell-spreading area along the PEG-DA concentration-gradient hydrogel. C) Quantification of the cell-spreading area in the gradient region (*** p < 0.05).

Microfluidic Device: The microfluidic device consisted of a PDMS mold with a straight fluidic channel (50 mm × 2.0 mm × 100 μm) and a bottom glass slide [29]. The PDMS mold was fabricated using standard soft-lithography methods and reversibly sealed to the glass coverslip. An inlet and outlet of the microchannel were created by a sharp punch (hole radius: 0.4 mm). The glass slide was pre-treated with 3-(trimethoxysilyl) propyl methacrylate (TMS-PMA) to create free methacrylate groups on the glass surface, which promoted the adhesion of PEG-DA hydrogels after UV lithography methods and reversibly sealed to the glass coverslip. An inlet and outlet of the microchannel were created by a sharp punch (hole radius: 0.4 mm). The glass slide was pre-treated with 3-(trimethoxysilyl) propyl methacrylate (TMS-PMA) to create free methacrylate groups on the glass surface, which promoted the adhesion of PEG-DA hydrogels after UV exposure.

Generation of Chemical Gradient Hydrogels: The microchannel was initially introduced with the pre-filling solution, namely Dulbecco’s Phosphate Buffered Saline (DPBS, Gibco, Carlsbad, CA) or a low-concentrated PEG-DA solution. Pre-filling solution (200 μL) was pipetted onto the outlet opening and PEG-DA solution (2 μL) with chemical molecules of interest or higher PEG-DA concentration was dropped onto the inlet opening. The difference of the surface tension between the two drops induced the automatic flow from inlet to outlet. Three drops of PEG-DA solution (20 wt%, 2 μL) containing Acr-PEG-RGDS (8.0 mM) were introduced into the inlet. HUVECs were cultured in endothelial cell basal medium (EBM-2, Clonetics) supplemented with vascular endothelial growth factor (VEGF, 0.5 mM), hydrocortisone (0.2 mM), epidermal growth factor (rHEGF, 0.5 mM), ascorbic acid (0.5 mM), r-human fibroblast growth factor-B (rhFGF-B, 2.0 mM), heparin (0.5 mM), recombinant long R insulin-like growth factor (R1-IGF, 0.5 mM) and gentamicin sulfate amphotericin-B (GA-10000, 5 mL) at 37 °C in a humidified incubator. Upon trypsinization, the cells were seeded on the surface of the hydrogels with Acr-PEG-RGDS gradient, which were pre-washed five times in DPBS and twice in EBM-2. The cell seeding density was 4 × 10^5 cells cm^-2. After 6 h of incubation, the hydrogels were rinsed three times with sterile PBS to wash away unattached cells and then visualized by counting the attached-cell number in a minimum of eight images from three individual hydrogels.

Cell Study on PEG-DA Concentration-Gradient Hydrogel: To create the PEG-DA concentration-gradient hydrogel for cell study, a PEG-DA solution (5 wt %) was first dropped into the microchannel and three drops of a PEG-DA solution (20 wt%, 2 μL) containing Acr-PEG-RGDS (8.0 mM) were introduced into the inlet. To promote the adhesion of HUVECs onto the PEG-DA concentration-gradient hydrogel, Acr-PEG-RGDS (5.0 mM) was incorporated into both solutions. HUVECs were seeded onto the hydrogels at a density of 1 × 10^5 cells cm^-2. After 24 h of incubation, the cells were fixed for 15 min in a glutaraldehyde solution (0.75% in PBS), and then observed under a phase-contrast microscope. The effect of PEG-DA concentration on the cell morphology was investigated.

Statistical Analysis: All quantitative data is expressed as mean ± standard deviation. Statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey HSD tests for post-hoc comparison using the SPSS 14.0 statistical package (SPSS Inc. Chicago, USA). Values of p < 0.05 were considered statistically significant.

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