Published in final edited form as:

Lab Chip. 2008 July ; 8(7): 1056–1061. doi:10.1039/b804234a.

Stop-Flow Lithography to Generate Cell-Laden Microgel Particles

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Abstract

Encapsulating cells within hydrogels is important for generating three-dimensional (3D) tissue constructs for drug delivery and tissue engineering. This paper describes, for the first time, the fabrication of large numbers of cell-laden microgel particles using a continuous microfluidic process called stop-flow lithography (SFL). Prepolymer solution containing cells was flowed through a microfluidic device and arrays of individual particles were repeatedly defined using pulses of UV light through a transparency mask. Unlike photolithography, SFL can be used to synthesize microgel particles continuously while maintaining control over particle size, shape and anisotropy. Therefore, SFL may become a useful tool for generating cell-laden microgels for various biomedical applications.

1. Introduction

The use of polymeric biomaterials in tissue engineering has grown tremendously over the past few decades due both to their physical and chemical properties and to their biocompatibility.¹ Polymeric hydrogels, which are highly hydrated cross-linked polymer chains, are particularly attractive for engineering 3D tissue constructs due to their high water content.^{2–5} Cells can be encapsulated directly in hydrogels, which can be synthesized from both natural and synthetic polymers, and thus the cell seeding limitations associated with non-hydrogel tissue scaffolds can be circumvented. Microengineered hydrogels can potentially be applied in tissue engineering to recreate the complexities of *in vivo* tissue constructs either by engineering the microvasculature and cellular organization in large microscale scaffolds, or by assembling the building blocks in the shape of microgel tissue units to generate larger structures.^{5,6} A number of microfabrication techniques have been developed both for micropatterning hydrogels for cell encapsulation^{2,7–19} and for creating cell-laden microgel particles with controlled sizes and shapes.^{3,20,21} Micropatterning of hydrogels is typically carried out using either soft lithography or photolithography, and suffers from the limitation that the resulting hydrogels are usually stuck to the glass surfaces on which they are fabricated. Microgel particles which are free floating and can be assembled at will to generate tissue structures^{3,21} would provide a more desirable system for these constructs. We present here an approach to achieve this goal.

Poly(ethylene glycol) (PEG) is an inert biomaterial, which has been used extensively for the encapsulation of a diverse array of cell types such as chondrocytes,²² vascular smooth muscle cells,²³ osteoblasts²⁴ and mesenchymal stem cells,²⁵ in addition to its many uses in a broad array of biomedical applications.^{2–4,9–11,26,27} When PEG macromers are terminated with methacrylate or acrylate groups, they undergo rapid crosslinking on exposure to UV light in the presence of appropriate photoinitiators,^{26,28,29} and as such, can be used with standard photolithographic processes. This method has been used widely to control cell–microenvironment interactions in generating tissue engineered constructs that mimic native tissue architecture and direct cellular differentiation and organization.^{3,5} A shortcoming of photolithography is that it is a batch process which usually limits it to have low yields. Further, photolithography is difficult to perform on prepolymer solutions used for encapsulating cells due to their low viscosities.

Another lithography system, called continuous flow lithography (CFL), has recently been developed that can produce microengineered hydrogels continuously and work on a variety of different materials.³⁰ However, CFL is not suitable for tissue engineering applications, since it requires a short polymerization time or slow flow-rate, in order to avoid smearing of the patterned feature in the hydrogel. Therefore, highly concentrated (either monomer or photoinitiator) prepolymer solutions are required which would be toxic to cells.³¹

To overcome the limitations of CFL, SFL has been developed.³² In the current work, we use SFL to synthesize large numbers of cell encapsulated hydrogels in a continuous manner. SFL provides distinct advantages over CFL in both throughput and control over shape and size.³³ We use SFL with photocrosslinkable prepolymer solutions containing cells, which are flowed through microfluidic channels. Cells are permanently encapsulated within PEG microgel particles by exposure to UV light and then flowed out of the device. Compared to standard lithography, SFL provides more flexibility in the type of materials and allows the use of co-flowing streams to generate particles with several adjacent functionalities.^{30,32,33} This technique has previously been used for the synthesis of multi-functional encoded particles for bimolecular analysis.³⁴ In this work, we demonstrate the use of this technique for the encapsulation of cells in polymeric particles of desired shapes. We also characterize the viability of cells in hydrogels created using SFL.

2. Experimental

2.1. Cell culture

Cells were manipulated under sterile tissue culture hoods and maintained in a 95% air/5% CO₂ humidified incubator at 37 °C. NIH-3T3 mouse fibroblast cells were maintained in cell culture media composed of Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. Confluent dishes of NIH-3T3 fibroblast cells were passaged and fed every 2–3 days.

2.2 Photolithography

Materials—Solutions containing 10–40% (w/v) poly(ethylene glycol) diacrylate (PEGDA, 700 MW, Sigma) in culture media were prepared for the experiments. Prior to UV photopolymerization, 1–5% photoinitiator (w/v), 2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (Irgacure 2959, CIBA Chemicals) was added to the prepolymer solution. 0.3% (w/v) n-vinyl pyrrolidone (NVP) was also added to accelerate the photoinitiation reaction in selected samples.

Microgel batch polymerization—A NIH-3T3 fibroblast cell pellet was suspended in photocrosslinkable PEGDA prepolymer solution (1.5 million cells mL⁻¹). After mixing, 8–10

μL of this solution was placed on top of a cover glass slide (Figure 1a) and exposed 12.4 mW/cm² UV light (360–480 nm) for various periods of time (Figure 1a). Following UV exposure, the cover slide was removed carefully, placed into culture media, and incubated 37 °C for 60 minutes. Hydrogel microblocks were made from 10–40% PEGDA, 1–5% Irgacure 2959 (I2959), and 0–0.3% NVP. The shape and size of these hydrogels were controlled using photomasks. In addition, the thickness (150 μm) of the hydrogels was controlled by the height of the spacers that were placed on two edges of the glass slide.

2.3 Stop-Flow Lithography (SFL)

Materials—In the SFL experiments (Figure 1b), the prepolymer was mixture of 20% (w/v) PEGDA (700 MW), 4% (w/v) I2959, 75.7% culture media, and 0.3% (w/v) NVP.

Microfluidic device—Microfluidic devices were fabricated by pouring polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) on patterned silicon wafers (SU-8 photoresist, Microchem) containing positive-relief channels.³² In all our experiments, we used straight channels with a height of 35 μm and a width of 500 μm . The PDMS-based microfluidic device was peeled off from the wafer and an inlet port was punched into the device to enable the prepolymer solution to be introduced to the channel. An outlet reservoir for collection of the hydrogel particles with their encapsulated cells after polymerization was cut out from the device at the other end of the channel. The PDMS microfluidic devices were bonded to PDMS coated glass slides using oxygen plasma. These assemblies were mounted on an inverted microscope (Axiovert 200, Zeiss) and the formation of cell-encapsulated hydrogels was visualized using a CCD camera (KP-M1A, Hitachi). Images were analyzed and processed using NIH Image software.

Cell encapsulation using SFL—The prepolymer solution containing cells (6×10^6 cells per mL) was passed through a microfluidic channel using the SFL setup (Figure 1b). The SFL process essentially involves the three steps of stopping the liquid flow, polymerizing the patterned solution, and flowing of the particles out of the device.³² The composition of the prepolymer solution used in the SFL hydrogel syntheses was determined from the optimal conditions obtained for the traditional photolithography process. This prepolymer solution with cells was polymerized by a flash of UV light from a 100 W HBO mercury lamp through a photomask. The photomask was designed using AutoCAD and was printed on a high-resolution printer at CAD/ART Services (Bandon, OR). A filter set that provides wide UV excitation (11000v2: UV, Chroma) was used to filter out the undesired wavelengths. A typical exposure time of 800 ms and a pressure of 1.2 psi were used for all experiments. The microgels with encapsulated cells were collected in the outlet reservoir, which was filled with the culture media to avoid aggregation of the microgel particles and to ensure that microgels with encapsulated cells were not exposed to a high concentration of the cytotoxic monomer solution in the reservoir.

2.4 Cell Viability Measurements

Cell viability was determined using a live/dead assay (Invitrogen, CA) containing calcein AM (live cells, green) and ethidium homodimer (dead cells, red). Cell-laden microgels were incubated for 1 hour after fabrication following which they were stained by incubation with the live/dead assay agents for 10 minutes to allow the stain to diffuse into the hydrogels. The stain was removed by washing the hydrogels with culture media before the hydrogels were imaged. Cell viability was analyzed five times for the photolithography system, and three times for SFL. The fraction of viable cells in 400 μm square hydrogels prepared by photolithography (Figure 2) and in 120 μm diameter circular hydrogels prepared by SFL (Figure 5) was quantified.

3. Results and Discussion

Hydrogel microblocks were fabricated via both traditional photolithography and SFL. Standard photolithography was performed first in order to quickly screen for conditions that are both suitable for SFL and yield high cell viabilities. Monomer and photoinitiator concentrations, and polymerization time were systematically varied. These parameters have been shown in previous studies to influence cell viability inside photocrosslinked hydrogels significantly.^{28, 35} Our goal was to maximize cell viability in the hydrogels by determining the optimized prepolymer composition and crosslinking parameters using photolithography.

The synthesis of microgels from photocrosslinkable monomers requires UV light, photoinitiator, and PEGDA, each of which is known to influence the viability of cells negatively when used at concentrations higher than a threshold, the threshold being different for different polymers and photoinitiators which needs to be determined experimentally. Thus, to maximize cell viability, it is desirable to minimize the UV exposure for a specific concentration of PEGDA and photoinitiator and yet still ensure a fully formed hydrogel, i.e., to maintain microgel pattern fidelity. The minimum exposure time required to fabricate hydrogels with controlled features was determined for a range of prepolymer solutions by systematically varying the PEGDA and I2959 concentrations. Overexposure of the prepolymer solutions to UV radiation resulted in the formation of hydrogels larger than the mask features while under exposure produced gels that were smaller than these features. The optimum UV exposure time was deemed to be the time to produce microgels with no distortion in their shapes (Figure 3).

The maximum allowable UV exposure in SFL, is limited by the stability of the masks themselves. An exposure time of 1.5–2 s can burn the standard polymeric photomasks used in SFL and is typically avoided. Further, at long exposure times, there is the possibility of free-radical diffusion outside the intended polymerization region that compromises pattern fidelity in the hydrogels formed. To avoid damage to our masks and to ensure perfect control over shape and size, exposure times of less than 1 s were used in the SFL system. An exposure time of 1 s for SFL in a 35 μm tall channel corresponds to a time of about 5.5 s for creating extruded square shaped hydrogels ($400 \times 400 \times 150 \mu\text{m}$) using photolithography as determined experimentally. This difference in time is due to the difference in light intensity of the UV lamps used and thickness of particles formed in each system

The cell viabilities obtained in the photolithography experiments at different concentrations of PEGDA (10–40%) and at a constant I2959 concentration of 0.5%, are shown in Figure 3a. Cell viabilities at PEGDA concentrations of 10% and 20% were found to be high (>80%), but decreased with increasing concentration beyond 20%. Although cell viabilities at 10% and 20% PEGDA were high, the exposure times required to crosslink the gels were not suitable for use in SFL. The concentration of I2959 was, therefore, increased while keeping the PEGDA concentration at 20%. The cell viabilities decreased with increasing I2959 concentrations at a PEGDA concentration of 20% as shown in Figure 3b. The minimum UV exposure time also decreased with increasing I2959 concentration (6.5 s at a concentration of 5%), but again not sufficiently to be of use in SFL.

The UV exposure time was further decreased without increasing the concentrations of PEG or I2959 by adding NVP to the prepolymer solution. NVP has been shown to accelerate reactions involving acrylate groups,³⁶ with a high cell viability retention when used at low concentrations.³⁷ Hydrogels formed from prepolymer treated with 0.3% NVP (w/v) showed similar viabilities at different I2959 concentrations (1–4%) as indicated in Figure 3b. The exposure times obtained using NVP are significantly lower than those obtained without NVP and are suitable for use in SFL at an I2959 concentration of 4%. A suitable prepolymer

composition for use in SFL is 20% PEGDA, 4% I2959 and 0.3% NVP, as the exposure time required for pattern fidelity is low enough for use in SFL while ensuring reasonable cell viability. In Figure 3 we reported the cell viability and exposure times for the combinations of PEGdA and I2959 with/without NVP analyzed in this work. The screening can be carried out for other combinations of polymers and photoinitiators and may result in a higher cell viability.

We analyzed the suitability of the proposed prepolymer solution in the SFL polymerization of hydrogels of different shapes using appropriate photomasks as shown in Figure 4. SFL cycles through three states which each have associated times: the time to stop the flow (t_{stop}), polymerization time ($t_{\text{polymerize}}$), and a time required to flush particles out of view (t_{flow}). The typical values for t_{stop} , $t_{\text{polymerize}}$, and t_{flow} in SFL were 200 ms, 800 ms and 100 ms, respectively, resulting in a throughput of $\sim 10^3$ particles h^{-1} using a mask which patterns one particle per exposure. For microgel particles with dimensions of $100\text{ }\mu\text{m}$ in a $35\text{ }\mu\text{m}$ tall channel, as synthesized here, the rate of particle generation by SFL can be increased by an order of magnitude by using photomasks which pattern multiple particles per exposure.³² Further, several microfluidic channels can be combined in parallel, making the fabrication potential of SFL greater than that of photolithography, while maintaining precise control over the shape and size of each gel microparticle. A high rate of hydrogel production is essential to be able to produce sufficient quantities of particles for applications such as the self-assembly of these hydrogels into tissue structures. A circular hydrogel generated using SFL and its corresponding live/dead image is shown in Figure 5. The cell viability measured in hydrogels generated using SFL ($68 \pm 3\%$) correlated well with our values predicted using information from the photolithography system. Therefore, SFL holds great promise for the high-throughput generation of cell-laden microgels which can be used for a variety of diagnostic tools in drug delivery, DNA sequencing, and tissue engineered constructs.^{3,38–40}

The viability can likely be further optimized if necessary for different biomedical applications. The decreased viability was caused by the unfavorable conditions of the prepolymer solution, particularly, the high concentration of I2959. This high photoinitiator concentration was required due to the time constraint imposed by the masks used. To eliminate the necessity of a prepolymer that crosslinks rapidly, glass or chrome masks can be used which can withstand longer exposure times. Further, other more potent combinations of polymers and photoinitiators which require lower polymerization times while ensuring high cell viability can be investigated. These combinations of polymers and photoinitiators can be screened using photolithography to minimize exposure times before being used in SFL. Shorter exposure times would alleviate diffusion limitations to maintaining shape fidelity and increase the throughput.

The SFL system can be potentially used for cells other than the NIH-3T3 mouse fibroblast cells used in this work. The hydrogel particles synthesized in this study could be assembled into ordered meso-structures using various methods such as evaporation driven assembly,⁴¹ DNA hybridization,^{42–44} or using selective wetting at a liquid-liquid interface.^{45,46} Furthermore, SFL can be used for creating multicomponent hydrogels^{30,32–34} by co-flowing more than one stream containing either different cells and the same prepolymer or the same cells but different prepolymers or a combination of both. These anisotropic particles can be assembled, exploiting the difference in surface energies of different regions of a particle, finding potential application for generating tissue constructs. Another application of anisotropic microgel particles is multi-cell drug assays, reducing the cost and time involved significantly. The hydrogels formed using SFL can also be used for immuno-isolation of cells for implantation.^{14–19}

4. Conclusion

We demonstrated the use of SFL to generate high-throughput cell-laden hydrogel microblocks in a continuous manner. The viability of cells encapsulated within hydrogels, for different

experimental parameters (i.e. monomer and photoinitiator concentrations), was analyzed using a photolithography setup, to obtain an optimized prepolymer solution for SFL. The majority of cells encapsulated within hydrogels generated by SFL using the optimized solution remained viable. The potential of SFL to create shape-controlled microgels continuously makes hydrogel generation by SFL a fertile field for further studies. A comprehensive characterization of hydrogels generated by SFL with respect to nutrient transfer in and out of the hydrogels might reveal that SFL is indeed a promising technique for the generation of cell-encapsulated hydrogels for the fabrication of 3D tissue constructs.

Acknowledgments

This paper was partly supported by the Coulter Foundation, National Institutes of Health (NIH), the Center for Integration of Medicine and Innovative Technology (CIMIT), US Army Core of Engineer, the Charles Stark Draper Laboratory and the NSF NIRT Grant No. CTS-0304128. S. Ali thanks the Higher Education Commission (HEC) of Pakistan for financial support and we thank Dhananjay Dendukuri and Daniel C Pregibon for useful discussions.

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Figure 1. Schematic of the photolithography and SFL processes. (a) Schematic drawing of the formation of hydrogels using photolithography. Cells were encapsulated in hydrogels by exposing the prepolymer to UV light through a photomask. (b) Schematic diagram for the formation of cell-laden microgels using SFL. A prepolymer solution containing cells is flowed through a microchannel and polymerized by UV light through a photomask and a microscope objective.

Figure 2. Cells encapsulated within a PEGDA hydrogel unit fabricated by using photolithography. (a) Phase contrast image of a hydrogel microblock. This microgel unit was made by using a prepolymer solution of 20% PEGDA, 4% Irgacure, and 0.3% NVP. (b) Corresponding fluorescent image for the cell viability expressed by calcein AM (live cells, green) and ethidium homodimer (dead cells, red). Scale bars are 100 μm .

Figure 3.

(a) Cell viability at different PEGDA concentrations with 0.5% I2959 using the photolithography system. The viability was high at 10% and 20% PEGDA, although long UV exposure times (>25 s) were required. The unpaired Student's t-test was performed to ascertain the statistical significance of the variations in cell viability for the data sets obtained at different PEGDA concentrations. The data sets for 20% and 30% PEGDA (*) gave a p-value < 0.05 and the data sets for 20% and 40% PEGDA (**) gave a p-value < 0.01, indicating that the variation in cell viability was statistically significant in both cases and thus, an increase in PEGDA concentration from 20% to 30% and subsequently 40%, results in decreasing cell viability. Therefore, a PEGDA concentration of 20% was used in subsequent studies that varied

photoinitiator concentration. (b) Viability at different I2959 concentrations with 20% PEGDA in a photolithography system. A linear decrease was observed for samples without NVP treatment, while the cell viability of samples treated with NVP was similar at different I2959 concentrations (1~4%). The optimized exposure times are marked above the corresponding conditions in the histogram.

Figure 4.

Cells encapsulated within hydrogels using SFL. (a) Cell-laden hydrogel micro-blocks at the outlet reservoir. (b–d) Cells trapped within hydrogels of different shapes. Scale bar in (a) is 100 μm . Scale bars in (b), (c), and (d) are 20 μm .

Figure 5.

Cells encapsulated within PEGDA hydrogel units fabricated by using SFL. (a) Phase contrast image of a hydrogel microblock. This microblock unit was made by using a prepolymer solution of 20% PEGDA, 4% I2959, and 0.3% NVP. (b) Fluorescent image for the cell viability expressed by calcein AM (live cells, green) and ethidium homodimer (dead cells, red). Scale bars are 50 μm .