

## Poly(ethylene glycol) Hydrogel Microstructures Encapsulating Living Cells

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We present an easy and effective method for the encapsulation of cells inside PEG-based hydrogel microstructures fabricated using photolithography. High-density arrays of three-dimensional microstructures were created on substrates using this method. Mammalian cells were encapsulated in cylindrical hydrogel microstructures of 600 and 50  $\mu\text{m}$  in diameter or in cubic hydrogel structures in microfluidic channels. Reducing lateral dimension of the individual hydrogel microstructure to 50  $\mu\text{m}$  allowed us to isolate 1–3 cells per microstructure. Viability assays demonstrated that cells remained viable inside these hydrogels after encapsulation for up to 7 days.

Here, we describe the fabrication using photolithography of poly(ethylene glycol) (PEG)-based hydrogel microstructures encapsulating viable mammalian cells on glass and silicon substrates. Cell-based biosensing devices for applications such as high-throughput drug screening require the accurate positioning of cells into arrays that can be addressed (preferably using optical methods) and integrated with microfluidic channels for sample introduction.<sup>1</sup> Much research has been conducted in the area of cell patterning using chemical or lithographic methods for the spatial control of cell adhesion and growth.<sup>2–9</sup> In most of these applications, anchorage-dependent cells are immobilized on a two-dimensional substrate. However, in a two-dimensional system, nonadherent cells are difficult to immobilize and adherent cells such as fibroblasts and hepatocytes are in an unnatural environment; i.e., in tissue they exist in a three-dimensional hydrogel matrix consisting of proteins and polysaccharides (i.e., the extracellular matrix). As a result, the response of these cells to drug candidates may be very different than that of the same cells in their native tissue.

One strategy to overcome the problems associated with a two-dimensional culture system is to encapsulate cells inside a three-dimensional hydrogel matrix.<sup>10,11</sup> Originally, cell encapsulation technologies using hydrogels were

developed for tissue engineering or therapeutic cell transplantation to prevent the rejection of transplanted cells by the host's immune system. Hydrogels have been widely used because of their high water content, softness, pliability, biocompatibility, and easily controlled mass transfer properties,<sup>12–14</sup> essential for allowing transport of nutrients to and waste products from cells. In one such example of encapsulating cells in hydrogels for the potential treatment of type I diabetes, photopolymerized poly(ethylene glycol)-based hydrogels were developed for encapsulating islet cells without loss of cell viability or insulin secretion.<sup>15–17</sup> In other research related to chemical sensor development, photolithographically patterned hydrogels were used in the development of both electrochemical and optical sensors.<sup>18–21</sup> More recently, Beebe and colleagues fabricated hydrogel structures inside microfluidic channels for use as "smart" flow controllers.<sup>22,23</sup>

To form cell-containing hydrogel microstructures on surfaces, we modified the surfaces of glass and silicon substrates to promote good adhesion, essential for the gel to remain stationary in a flow field. The substrate surface was modified with an organosilane to create surface-tethered methacrylate groups capable of covalent bonding with hydrogel during photopolymerization.<sup>21</sup> Substrates were first immersed in "piranha" solution consisting of 3:1 ratio of 30% w/v  $\text{H}_2\text{O}_2$  and  $\text{H}_2\text{SO}_4$  to clean and hydroxylate the substrate surface. The hydroxylated

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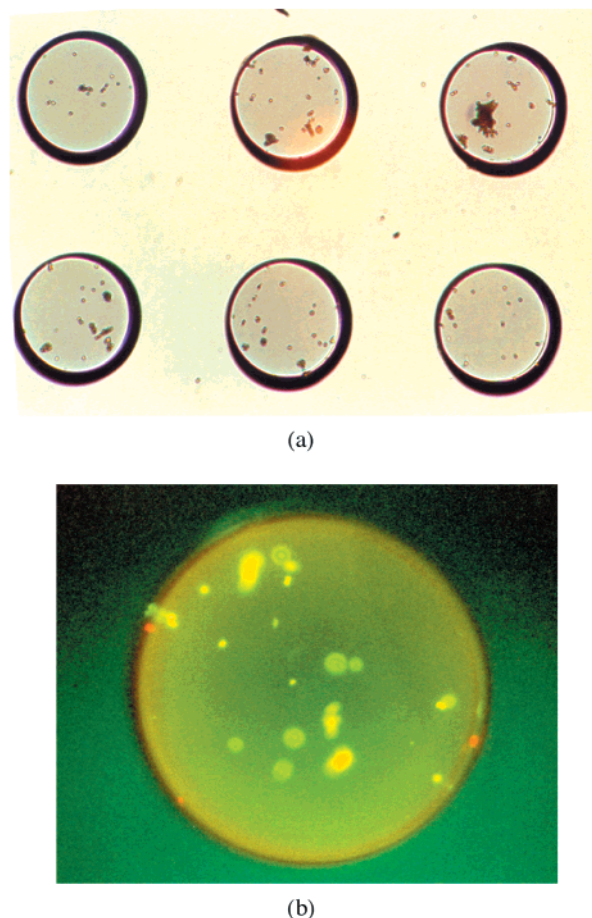
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surface was then immersed for 5 min in a 1 mM solution of 3-(trichlorosilyl)propyl methacrylate (TPM, Sigma-Aldrich, St. Louis, MO) in 80%/20% (v/v) mixture of heptane/carbon tetrachloride, which resulted in formation of a dense network of Si–O–Si bonds on the substrate surface and pendant methacrylate functionalities at the substrate/solution interface as confirmed by time-of-flight secondary ion mass spectrometry (TOF-SIMS).<sup>21</sup> This surface modification was easily visualized by the increase in water contact angle associated with hydrophobic methacrylated alkylsilanes on hydrophilic SiO<sub>2</sub>. Ellipsometry measurements of modified Si/SiO<sub>2</sub> surfaces indicated that the organosilane films were  $14 \pm 3$  Å thick, indicating the presence of a monolayer of TPM on the substrate surface.

Hydrogel microstructures encapsulating murine 3T3 fibroblasts were fabricated using proximity photolithography. A PEG-diacrylate (PEG-DA, MW 575, Sigma-Aldrich, or MW 4000, Polysciences, Warrington, PA) precursor solution containing 0.5% (w/w) Darocur 1173 (1-phenyl-2-hydroxy-2-methyl-1-propanone, Ciba Specialty Chemicals, Tarrytown, NY) as a photoinitiator was mixed with a cell suspension in cell culture media to produce a cell density about  $4$  to  $5 \times 10^6$  cells/mL in the gel precursor solution. Fibroblasts were cultured on tissue culture polystyrene in Dulbecco's modified Eagle media (DMEM with 4.5 g/L glucose and 10% FBS, Sigma-Aldrich) and incubated at 37 °C in 5% CO<sub>2</sub> and 95% air until near confluence. Cells were detached from culture flasks by trypsinization with 0.25% trypsin and 0.13% EDTA in phosphate-buffered saline. Cells were transferred back to cell culture media and then added to the gel precursor solution. The cell-containing polymer suspension was spin-coated onto functionalized substrates at 1500 rpm for 10 s to form uniform fluid layer. This layer was covered with a photomask and exposed to 365 nm UV light (300 mW/cm<sup>2</sup>) for 0.5 s to 30 s through the photomask. Upon exposure to UV light, only exposed regions underwent free-radical-induced gelation and became insoluble in common PEG solvents such as water. As a result, desired microstructures were obtained by washing away unreacted precursor solution with PBS or cell culture medium so that only the hydrogel microstructures remained on the substrate surface. During the UV-light-induced gelation process, cells suspended in the polymer precursor solution were encapsulated in the resultant hydrogel microstructures. Serum proteins present in the precursor solution were also likely entrapped in the gel to some extent. After encapsulation, surfaces with cell-containing microstructures were immersed in cell culture media (DMEM with 10% fetal bovine serum) and incubated in a 5% CO<sub>2</sub> atmosphere at 37 °C to assess viability.

Methacrylate moieties on the substrate surface also participate in the free radical polymerization and create covalent bonding between acrylate groups present in the bulk gel and those on the surface, thus fixing the hydrogel structures to the substrate. Long-term adhesion of cell-containing hydrogel arrays to a silicon surface was verified by placing hydrogel elements into an aqueous environment for over a week at ambient temperature. Upon hydration, PEG hydrogels may expand in volume by over 100%.<sup>24</sup> In the absence of covalent attachment to the substrate, the mechanical forces associated with swelling are sufficient to cause the gels to delaminate from the surface. Here, the TPM monolayer binds the gel to the surface and prevents delamination while still allowing the gel to swell



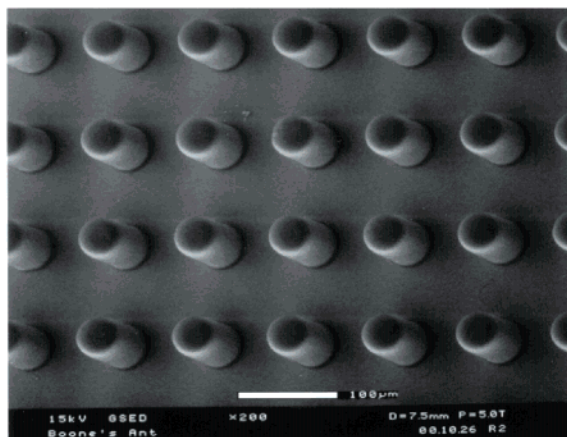
**Figure 1.** A 600  $\mu\text{m}$  diameter PEG hydrogel microstructures on a glass substrate: (a) optical bright-field micrograph of hydrogel microstructures containing 3T3 fibroblasts; (b) LIVE/DEAD fluorescent viability assay indicated the encapsulated cells were viable after 24 h in the hydrogels, as evident by the green color. Dead cells in this assay stain red as seen for three cells on the periphery of the microstructure.

with aqueous media. However, the bound gel tends to swell anisotropically, i.e., the dimensions at the base of the gel do not change but rather the gel swells upward away from the surface.<sup>21</sup> Here, the gels were fabricated at approximately their equilibrium water content because of the aqueous cell culture media added along with the cells. Thus, the gels would not physically swell with additional water. However, covalent attachment of the gels to the substrate surface was still necessary as unattached gels were easily washed from the surface.

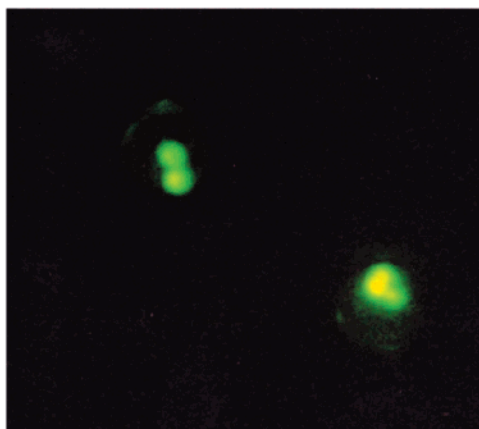
To optimize the size of the cell-containing microstructures, various spin-coating rates were tested to create thicker gels and microstructures with greater aspect ratios. As expected, the thickness of the deposited layer of precursor solution was found to be inversely proportional to the spin rate<sup>21</sup> and thus allowed control over the height of hydrogel microstructures. Spin rates of 4000 rpm resulted in cylindrical hydrogels of about 10  $\mu\text{m}$  in height as measured by profilometry, while polymer layer spun at 1500 rpm yielded hydrogel elements about 70  $\mu\text{m}$  in height as observed by environmental scanning electron microscopy (ESEM). Hence, both lateral and vertical dimensions of hydrogel microstructures could be controlled, the former by feature size of the photomask (to a minimum size of 7  $\mu\text{m}$ <sup>21</sup>) and the latter by the spin-coating rate. By using masks with different feature sizes and using different spin-coating rates, we were able to create cell-

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(a)

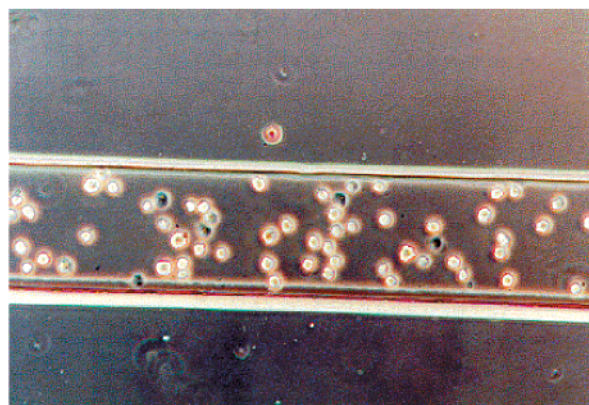


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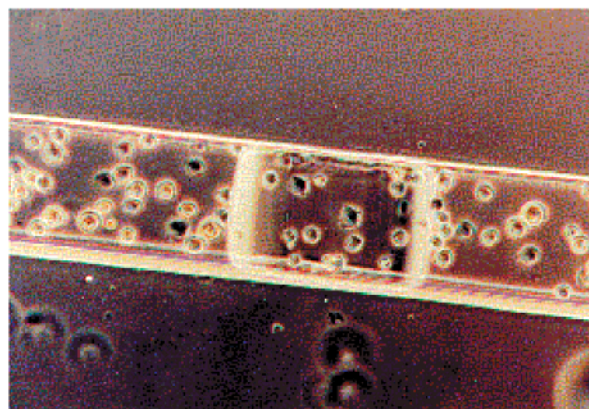
**Figure 2.** 3T3 fibroblast containing hydrogel microstructures of 50  $\mu\text{m}$  diameter cylinders on a silicon substrate: (a) ESEM micrograph of hydrogel microstructures; (b) LIVE/DEAD fluorescent viability assay indicating the presence of cells in the microstructures and their viability after 24 h in the hydrogels.

containing microstructures with aspect ratios ranging from 0.02 to 1.4.

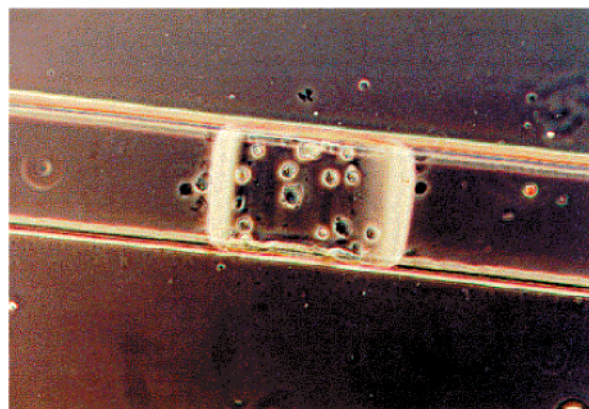
Figure 1a shows the optical bright-field micrographs of an array of 600  $\mu\text{m}$  diameter hydrogel microstructures containing mouse 3T3 fibroblasts. The cells were completely encapsulated within the microstructures with no cells or cell processes evident outside the gel. The transparent nature of PEG-based hydrogel allowed the observation of cells in the hydrogel structure through optical microscopy without staining. An approximately equal number of cells (30 per microstructure) were observed in each of the several hydrogel elements. Even though the size resolution of proximity lithography is larger than that of contact lithography, high-quality hydrogel microstructures of 50  $\mu\text{m}$  diameter were obtained as shown by the electron micrograph in Figure 2a. These cylindrical microstructures were of an obviously three-dimensional nature and were arranged in a  $20 \times 20$  square with 50  $\mu\text{m}$  spacing between elements so that as many as 400 microstructures could be reproducibly fabricated in a 2 mm<sup>2</sup> area. While 600  $\mu\text{m}$  hydrogel microstructures contained numerous cells, 50  $\mu\text{m}$  diameter microstructures had only 1–3 cells encapsulated per structure as seen in Figure 2b, with some microstructures absent of cells. In both types of microstructures, encapsulated cells appeared rounded even after 24 h but were found to spread slowly over the course of several days. The slow rate of spreading



(a)



(b)



(c)

**Figure 3.** A cell-containing hydrogel microstructure fabricated with a poly(dimethylsiloxane) microchannel: (a) a cell-containing hydrogel precursor solution in a microchannel; (b) gelation of the hydrogel inside the microchannel after exposure to UV light through a photomask; (c) a cell-containing hydrogel microstructure inside a microchannel after the removal of unreacted precursor solution.

by encapsulated cells was likely caused by insufficient protein in the gel, as PEG inhibits cell adhesion and proteins such as collagen are required for cell adhesion and spreading. Thus cells may not have spread until they themselves produced sufficient extracellular matrix.

As is apparent from Figure 2a, the fact that these microstructures contain cells is not readily evident by

electron microscopy because the cells are completely encapsulated within the gel. Indeed, even if cells were in the microstructures, the question remains as to whether they are viable or not. Cell viability was anticipated because UV polymerization conditions and chemical components were chosen to minimize cytotoxicity,<sup>25</sup> and the resulting gels possessed sufficient permeability to permit the transport of nutrients and oxygen to the cells.<sup>26</sup> The viability of individual cells in hydrogel microstructures with diameters of 600 and 50  $\mu\text{m}$  was investigated using LIVE/DEAD viability/cytotoxicity fluorescence assay (Molecular Probes, Inc., Eugene, OR) that stains live cells green and dead cells red. By use of this type of assay, cells within microstructures could be imaged and assayed for viability simultaneously. Figures 1b and 2b show stained fibroblasts encapsulated in these hydrogel microstructures after 24 h. As is evident by the green emitted light, approximately 80% of encapsulated cells were viable, demonstrating that the conditions for fabrication were sufficient for encapsulating viable cells in the photopolymerized PEG hydrogel. Cells encapsulated in PEG microstructures based on MW 575 PEG-DA lost viability after 3 days while those based on MW 4000 PEG-DA remained viable for 7 days. Murine SV-40 transformed hepatocytes were also encapsulated in a similar fashion and also retained viability (not shown).

As a control, cell-containing microstructures were incubated with 0.05% sodium azide in cell culture media. Azide anion killed the cells in the microstructures as anticipated and resulted in cells that stained red in the LIVE/DEAD assay. The viability of cells encapsulated in hydrogel was also measured using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.<sup>27</sup> In this assay, viable cells generated purple formazan crystals and confirmed that cells within the microstructures were viable.

On the basis of these results, we prepared cell-containing hydrogel microstructures inside microfluidic channels. Using well-established methods, an approximately 100

$\mu\text{m}$  wide, 50  $\mu\text{m}$  deep microchannel was created in poly-(dimethylsiloxane), treated in an  $\text{O}_2$  plasma to improve adhesion, and was sealed irreversibly to a glass slide to form an enclosed microchannel.<sup>28</sup> This microchannel was filled with a cell-containing hydrogel precursor solution (Figure 3a) and then exposed to UV light through a photomask. Only illuminated regions underwent photopolymerization and gelled inside the microchannel as shown in Figure 3b. Finally, by flushing the channel with PBS, we obtained the desired cell-containing hydrogel microstructure inside a microfluidic channel as shown in Figure 3c.

While more thorough investigations of how long the cells remain viable, the optimal conditions for sustaining cell survival inside the hydrogel microstructures, and the differences between phenotypes are needed, our preliminary results are very encouraging. While studies of cell patterning on two-dimensional substrates have used PEG to prevent cell adhesion, we have used patterned three-dimensional PEG hydrogels to encapsulate cells within the gel matrix. Future work will be focused on further investigation of cell viability and function with these gel microstructures and the formulation of gel chemistries designed to improve cell proliferation and function, perhaps through the inclusion of cell adhesion molecules such as collagen, fibronectin, vitronectin, or their peptide analogues. In addition, the microstructures presented here could be combined with a microfluidic device to create optical biosensor arrays of individually addressable single or multiple cell-containing hydrogel microstructures with potential applications in drug screening or pathogen detection.

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