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Cells in Micropatterned Hydrogels: Applications in Biosensing

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ABSTRACT

Here we will discuss the development of arrays of mammalian cells of differing phenotype integrated with microfluidics and microsensors for applications such as drug screening and used to monitor cellular effects of multiple chemical and biological candidates. To fabricate these arrays, we immobilized either single or small groups of cells in 3-dimensional poly(ethylene glycol) hydrogel microstructures fabricated on plastic or glass surfaces. These microstructures were created using either photolithography or printed using microarray robots. The resulting hydrogel microstructures were fabricated to dimensions as small as 10 microns in diameter with aspect ratios as high as 1.4. The gels were highly swollen with water to permit mass transfer of nutrients and potential analytes to the cells, and cell adhesion molecules were immobilized in the gel to allow cell attachment and spreading. Cell viability was confirmed using fluorescent assays and ESEM used to verify complete cell encapsulation. The specific and non-specific response of these cells to target molecules was monitored using optical or electrochemical detectors and analyzed to quantify the effect of these agents on the different phenotypes present in the array.

INTRODUCTION

Cell-based biosensing devices for applications such as high-throughput drug screening require accurate positioning of cells into arrays that can be addressed (preferably using optical methods) and integrated with microfluidic channels for sample introduction.[1-4] 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. In most of these applications, anchorage dependent cells are immobilized on a two-dimensional substrate. However, in a two-dimensional system, non-adherent 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 other cells, proteins, and polysaccharides. As the 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. Originally cell encapsulation technologies using hydrogels were developed for tissue engineering or therapeutic cell transplantation to prevent rejection of the transplanted cells by the host's immune system.[5-7] Hydrogels have been widely used because of their high water content, softness, pliability, biocompatibility, and easily controlled mass transfer properties, essential for allowing the transport of nutrients to and waste products from the

cells.[8, 9] In this paper, we described the fabrication using photolithography of poly (ethylene glycol) (PEG)-based hydrogel microstructures encapsulating viable mammalian cells on glass or silicon substrates, which could be combined with microfluidic system for the cell-based biosensor application.

EXPERIMENTAL DETAILS

Functionalized silicon or glass substrates were prepared as described previously.[10] Briefly, square silicon wafers with area ranging from 0.25 in^2 to 1 in^2 were placed in 'piranha' solution consisting of 3:1 ratio of 30 % w/v H_2O_2 and H_2SO_4 for 1 min. This step was followed by immersion of silicon wafers in 1mM solution of 3-(trichlorosilyl)propyl methacrylate (TPM) in a 4:1 mixture of heptane/carbon tetrachloride for 5 min., at room temperature. As result, substrate surface became functionalized with self-assembled monolayers of TPM. After functionalization, silicon wafers were washed with ethanol and D. I. water.

Murine fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic/antimycotic in a 75cm^2 cell culture flask. Confluent fibroblasts were subcultured every 2 to 3 days by trypsinization with 0.25% (w/v) trypsin and 0.13% (w/v) EDTA. Cell culture was incubated at 37°C in 5% CO_2 and 95% air.

Hydrogel microstructures encapsulating murine 3T3 fibroblasts were fabricated using proximity photolithography. A sterilized poly(ethylene glycol) diacrylate or PEG-DA precursor solution containing 0.5% (w/w) Darocur 1173 as a photoinitiator was mixed with a cell suspension in PBS (phosphate buffered saline) to produce a cell density about 4 to 5 x 10^6 cells/mL in the gel precursor solution. The cell-containing polymer suspension was spin-coated onto functionalized substrates at 1500 rpm for 10 seconds to form uniform fluid layer. This layer was covered with a photomask and exposed to 365 nm UV light (300 mW/cm^2) for 0.5 seconds 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 in gelation process, cells present in the polymer precursor solution were encapsulated in the resultant hydrogel microstructures. After encapsulation, surfaces with cell-containing microstructures were immersed in DMEM with 10% fetal bovine serum and incubated in a 5% CO_2 atmosphere at 37°C for 24 hours prior to examination. As a control, cell-containing microstructures were incubated with 0.05% sodium azide in same cell culture medium. ESEM and bright field optical microscopy were used to observe pattern morphology and verify presence of cells in the hydrogel arrays.

A Live/Dead Viability/Cytotoxicity fluorescence assay was used to investigate the cell viability after encapsulation of cells in the hydrogel microstructure. This assay uses SYTO 10 and Dead Red as fluorophores to distinguish living cells and dead cells. SYTO 10 stains live cells green and Dead Red stains dead cells red. For this assay, 2 µL of two fluorophores were added to 1mL HEPES-buffered saline solution (HBSS) to make the staining solution. The staining solution was placed on the hydrogel microstructure and

incubated for 15 minutes in the dark, at room temperature. Viability of cells encapsulated in hydrogel arrays was examined with fluorescence microscope.

Microchannels in polydimethyl siloxane (PDMS) were obtained by curing 10: 1 mixture of PDMS prepolymer and curing agent against Si master which has a negative pattern of the desired microchannel defined with photoresist. After cured for 24 hr at 60°C, PDMS replica was peeled from master and oxidized in an oxygen plasma with glass slide for 1 min. Bringing the oxidized PDMS and glass slide into conformal contact resulted in irreversible seal and forms a enclosed microchannel. To make inlet and outlet port of microfluidic device, several holes were made in PDMS replica using 16-gauge needle and tubes were hooked up inside these holes.

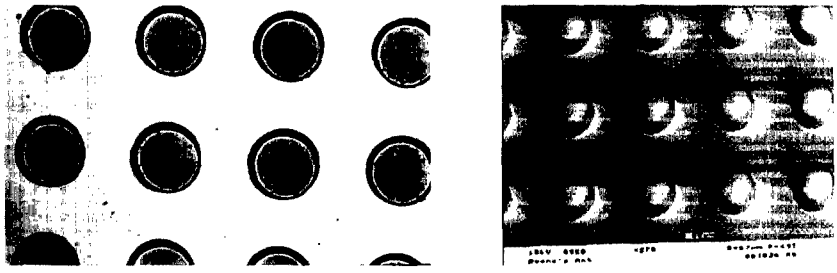
DISCUSSION

Cell encapsulation inside the hydrogel matrices is based upon free-radical polymerization of PEG-DA, a main constituent of the precursor solution. When exposed to UV light in the presence of photoinitiator, acrylate groups form unstable active sites which react with each other, thus creating insoluble, cross-linked, three-dimensional structures. Our group combined photopolymerization through photomask with surface modification to create surface bound, hydrogel structures of various geometries on SiO_2/Si substrates. It is hypothesized that 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 hydrogel structures to the substrate. Long term adhesion of cell-containing hydrogel arrays to silicon surface was verified by placing hydrogel elements into aqueous environment for over a week. Upon hydration, PEG hydrogels may expand in volume by over 100%. 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 with aqueous media. However, the bound gel tends 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. Here, the gels were fabricated with approximately their equilibrium water content because of the PBS 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 in an effort 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, and thus allowed control over the height of hydrogel microstructures. Spin-rates of 4000 rpm resulted in cylindrical hydrogels of about 10 μm in height as measured by profilometry, while polymer layer spun at 1500 rpm yielded hydrogel elements about 70 μm in height as observed by environmental scanning electron microscopy (ESEM). Hence, both lateral and height dimensions of hydrogel microstructures could be controlled, the latter by feature size of the photomask (to a minimum size of 7 μm)and the former by the spin-coating rate. By using masks

with different feature sizes and using different spin-coating rates, we were able to create cell-containing microstructures with aspect ratios ranging from 0.12 to 1.4.

Figure 1 (a) shows the optical bright-field micrographs of an array of 600 μm 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 us to observe cells in the hydrogel structure through optical microscopy and approximately an equal number of cells (30 per microstructure) were observed in each of the several hydrogel elements. Even though the image resolution of proximity lithography is larger than that of contact lithography, we were able to obtain high-quality hydrogel microstructures of 50 μm diameter as shown Figure 1 (b). These cylindrical microstructures were of an obviously three-dimensional nature and were arranged in a 20×20 square with 50 μm spacing between elements so that as many as 400 microstructures could be reproducibly fabricated in a 2 mm^2. As is apparent from Figure 1 (b) the fact that these microstructures contain cells is not readily evident by electron microscopy because the cells are completely encapsulated within the gel.



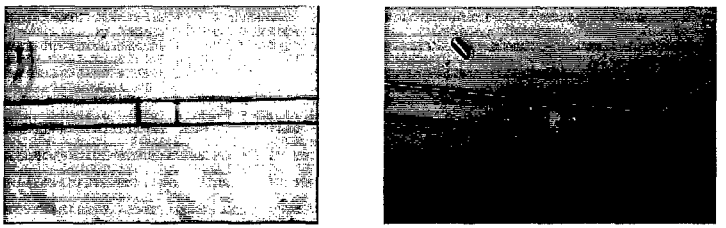
(a) (b)

Figure 1. Hydrogel microstructures on the glass substrate: (a) optical bright-field micrograph of 600 μm diameter hydrogel microstructures encapsulating 3T3 fibroblasts; (b) ESEM micrograph of 50 μm diameter hydrogel microstructures

Cells must survive and maintain their normal function after encapsulation. We investigated the viability of cells encapsulated in the circle hydrogel arrays with diameters of 600 μm and 50 μm using a LIVE/DEAD viability assay. As was evident by the green emitted light, encapsulated cells were viable in both arrays. While 600 μm hydrogel microstructures contained numerous cells, 50 μm diameter microstructures had only 1 to 3 cells encapsulated per structure, with some microstructures absent of cells. Cell viability was influenced by photopolymerization condition such as concentration of photoinitiator and intensity and exposure time of UV light. 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. In this assay, viable cells generated purple formazan crystals and confirmed that cells within the microstructures were viable.

Based on previous results, we prepared cell-containing hydrogel microstructures inside PDMS microfluidic channels. Recently, PDMS has been widely used as a material for the microfluidic system, because it is less expensive and less fragile than glass or Si/SiO_2. Furthermore, being based on replication, fabrication processes in PDMS are much faster and more convenient than those in glass or Si/SiO_2.

In our experiment, we first fabricated hydrogel microstructure inside an approximately 100 μm wide, 50 μm deep PDMS microchannel. Microchannel was filled with hydrogel precursor solution and then exposed to UV light through a photomask. Only exposed regions underwent photopolymerization and gelled inside the microchannel. Finally, by flushing the channel with PBS, we obtained the desired hydrogel microstructure inside a microfluidic channel as shown in Figure 2(a). To obtain cell-containing hydrogel microstructure inside microchannel, cell suspension in PBS was added to polymer precursor solution and this solution was injected to the microchannel. Figure 2(b) shows the cell-containing hydrogel microstructures inside a microfluidic channel fabricated by same procedure.



(a) (b)

Figure 2. Hydrogel microstructure fabricated in PDMS microchannel: (a) hydrogel microstructure without encapsulated cells; (b) cell-containing hydrogel microstructure inside microchannel.

CONCLUSIONS

In conclusion, we presented an easy and effective method for encapsulation of cells inside PEG-based hydrogel microstructures form using photolithography. High-density arrays of three-dimensional microstructures have been fabricated on substrates using this method. Cells were encapsulated in cylindrical hydrogel microstructures of 600 and 50 μm in diameter. Reducing lateral dimension of the individual hydrogel microstructure to 50 μm allowed us to isolate 1 to 3 cells per microstructure. Viability assays demonstrated that cells remained viable inside these hydrogels after encapsulation. While more thorough investigation of the optimal conditions for sustaining cell survival inside the hydrogel microstructures is needed, our preliminary results are very encouraging. Future work will be focused on further investigation of cell viability and function with these gels and the formulation of gel chemistries designed to improve cell function, perhaps through the inclusion of cell adhesion molecules in the gel formulation.

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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